MODULATION OF THE INTESTINAL MICROBIOME OF HYBRID TILAPIA (*OREOCHROMIS NILOTICUS* X. *O. MOSSAMBICUS*) USING ALTERNATIVE FEED INGREDIENTS

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Dedication Page

I would like to dedicate this dissertation to all of those who have helped me along my journey. To my teachers, particularly Mr. Mark Abert and Ms. Mona Franceschina, for inspiring a love of science in me and always pushing me to do my best. To my family, for their continued love and support through this journey, thank you for always being a sympathetic ear and motivating me to get this done. To my friends and loved ones, especially Andrew White and Jarmyn Munoz, thank you for reminding me to have fun through this stressful process and always believing in me.

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ABSTRACT

The intestinal microbiome is a dynamic ecosystem made up of bacteria, fungi, and protists, which together promote the overall health of the host. It regulates digestion and absorption of nutrients, prevents colonization of the intestinal tract by pathogenic organisms, drives the development of the host immune system, and even regulates chemical processes outside of the gastrointestinal tract, including the brain. Modulation of the intestinal microbiome can promote the growth and health of commercially important agriculture and aquaculture species in order to increase production and reduce costs. Probiotics, live beneficial bacteria incorporated in feed, and prebiotics, non-digestible feed ingredients, primarily inulin and oligosaccharides, are two of the primary modulation techniques used currently in aquaculture. Probiotics promote the colonization of the gut by the bacterial species of interest, while prebiotics promote the growth of beneficial bacteria by providing a substrate and feed source for the microbes. While there has been a lot of research on alternative feed ingredients to reduce cost and reliance on wild-caught fish in aquaculture feed, there are few studies on the effect of these feed ingredients on the intestinal microbiome. Many of these alternative ingredients are plant-based and thus provide a natural level of potential prebiotics that would modulate the intestinal microbiome of the aquaculture species. Tilapia (*Oreochromis sp.*) are the most widespread aquaculture species in the world due to their relative fecundity, omnivorous feeding habits, and tolerance of marginal growing conditions, making them ideal study species for alternative feed ingredients. The goal of this dissertation is to investigate the modulation of the intestinal microbiome of hybrid tilapia (*Oreochromis niloticus* x *O. mossambicus*) using alternative feed ingredients as potential prebiotics. Next generation sequencing was utilized to determine how the microbiome changes with alternative carbohydrate, protein, and lipid sources. The results of this work suggest that moringa leaf (*Moringa stenopetala)*, cassava (*Manihot esculenta*), and microalgae (*Arthrospira platensis* and *Schizochytrium limacinum*) can be included up to 12%, 26.2%, and 100%, respectively, without significantly slowing the growth parameters, making them acceptable feed alternatives. Additionally, the three alternative feed ingredients significantly altered the microbiome of hybrid tilapia.

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CHAPTER 1 INTRODUCTION

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Abstract

Tilapia (*Oreochromis sp.*) are the most widespread aquaculture species grown and are expected to continue increasing to meet the growing demand for fish by an ever-increasing human population. Fish health management is one of the primary concerns in the production of aquaculture species and a number of studies have been conducted to determine new procedures for reducing disease of aquaculture fishes, such as tilapia. The use of antibiotics as a preventative method is common practice in most aquaculture facilities; however, consumer demand and increasing incidence of antibiotic resistance have caused the reduction in the use of antibiotics being administered regularly. A more recent strategy is the incorporation of antibiotic alternatives, such as probiotics, prebiotics, and alternative feed ingredients to promote growth and health of the fish through the modulation of gastrointestinal microbiota. Intestinal autochthonous microbiota fulfils a number of important roles in host digestion, immunity, and intestinal integrity. In the past, the majority of studies on the intestinal microbiota of tilapia used culture-based methods that are not representative of the entire community. With the advancement of molecular techniques, current studies are utilizing culture-independent methods to monitor the microbial modulation in the tilapia gastrointestinal tract. This review discusses the effects of antibiotics, probiotics, prebiotics, and alternative feed ingredients on the intestinal microbiota of tilapia using culture-independent techniques. Though strides have been made in the understanding of tilapia intestinal microbiota, more research is needed into the microbial ecology, alternative feedstuff effects, and economic impacts of modulating intestinal microbiota of tilapia.

Introduction

Gastrointestinal (GI) microbiota are essential to their host's growth and survival (Burr, Gatlin, & Ricke, 2005; Cahill, 1990; Fouhy, Ross, Fitzgerald, Stanton, & Cotter, 2012; Sukanta K Nayak, 2010; Nicholson et al., 2012). They aid in digestion and energy homeostasis, prevent colonization of infectious agents, and help maintain the mucosal immunity of their host (Cahill, 1990; D. Merrifield & Ringø, 2014; S. K. Nayak, 2010; Nicholson et al., 2012). Increased research into the GI microbiota of mammalian vertebrates in the past decades has provided a clear picture of the benefits of GI microbiota and how microbial modulation can affect meat producing livestock species such as cattle, pig, and chicken (Hooper, Midtvedt, & Gordon, 2002;

Rajesh Jha & Berrocoso, 2016; Patterson & Burkholder, 2003; Richards, Gong, & de Lange, 2005; Taras, Vahjen, & Simon, 2007). However, research into the GI microbiota of aquaculture species has just recently increased.

The goal of aquaculture is to produce the highest quality meat for human consumption at the lowest possible cost. To meet this goal, modulation of the GI microbiota to promote growth and health of host (fish) can be accomplished through use of probiotics, prebiotics, and alternative feed ingredients in fish diets (Burr et al., 2005; Martínez Cruz, Ibáñez, Monroy Hermosillo, & Ramírez Saad, 2012; D. Merrifield & Ringø, 2014; S. K. Nayak, 2010; Sukanta K Nayak, 2010). Probiotics are defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance (Gibson & Roberfroid, 1995). Prebiotics are defined as non-digestible feed ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of healthful bacteria and by combating undesired bacteria in the intestinal tract (Gibson & Roberfroid, 1995). Alternative feed ingredients are unconventional feed ingredients usually derived from local sources and are rich in fibre content. The fibre content of alternative feed ingredients alters the GI microbiota in a manner similar to prebiotics. The benefits of probiotics, prebiotics, and alternative feed ingredients are well documented and their use in livestock animals and human health has increased in the past decades, leading to their recent expansion into aquaculture (Burr et al., 2005; Martínez Cruz et al., 2012; D. Merrifield & Ringø, 2014; Nicholson et al., 2012).

Fish health management is a primary concern in aquaculture to provide the maximum yield of a high-quality fish for human consumption (Burr et al., 2005). Historically, antibiotics were used to prevent the spread of disease in aquaculture facilities. However, recent concerns over the use of antibiotics in aquaculture led to the exploration and use of alternatives such as probiotics and prebiotics in fish diets to promote health and reduce the need for antibiotics (Defoirdt, Sorgeloos, & Bossier, 2011; Ferguson et al., 2010; L. Welker & Lim, 2011; Martínez Cruz et al., 2012; S. K. Nayak, 2010; Tuan, Duc, & Hatai, 2013).

Aquaculture feed is one of the primary costs in production, with fishmeal protein sources being the most expensive feed ingredient. A number of studies have been conducted to determine the optimal feed ingredients for growth and development of fishes, such as tilapia, at minimal cost (a-F. M. El-Sayed & Tacon, 1997; Mbahinzireki, Dabrowski, Lee, El-Saidy, & Wisner, 2001; D. Merrifield & Ringø, 2014; Naylor et al., 2009; Tri N. Nguyen, Davis, & Saoud, 2009; Poot-López,

Hernández, & Gasca-Leyva, 2014). However, very little information is available on the effects of either conventional or alternative feed ingredients on the intestinal microbiota of aquaculture species. Of those studies available, the vast majority utilized culture-dependent techniques that overlook the facultative anaerobic microbiota in the GI tract (Burr et al., 2005; Cahill, 1990; a-F. M. El-Sayed & Tacon, 1997; Vaughan et al., 2000). With the development of new molecular techniques, culture-independent studies of the GI microbiota of aquaculture species are providing new insights into this complex community (Sukanta K Nayak, 2010; Vaughan et al., 2000). Using these techniques, researchers can now study the effects of probiotics and prebiotics on the GI microbiota of commercially important fish species to determine the most beneficial feeding strategies (Dimitroglou et al., 2011; Martínez Cruz et al., 2012; D. Merrifield & Ringø, 2014; Sukanta K Nayak, 2010; Tuan et al., 2013). Alternative feed ingredients, in conjunction with probiotics and prebiotics, may provide an optimal environment for the growth of beneficial intestinal microbiota (a-F. M. El-Sayed & Tacon, 1997; Mbahinzireki et al., 2001; D. Merrifield & Ringø, 2014; Naylor et al., 2009; Tri N. Nguyen et al., 2009; Poot-López et al., 2014). These microbiota will influence the overall health and growth of tilapia, allowing for higher yields in aquaculture (Martínez Cruz et al., 2012; D. Merrifield & Ringø, 2014).

The term tilapia refers to a number of species in the *Cichlidae* family; however, for the purpose of this review we will focus on the three most common aquaculture species; Nile tilapia (*Oreochromis niloticus*), Mozambique tilapia (*O. mossambicus*), and the hybrid tilapia (*O. niloticus* × *O. aureus)*. The farming of tilapia is the most widespread type of aquaculture in the world (FAO, 2016) and they continue to increase in popularity due to their relative ease of cultivation and their mild flavour. Production of tilapia in the year 2012 exceeded 4.5 million tons worldwide with a value of over $7.6 billion USD and subsequently continues to increase (FAO, 2014). The ability to maximize production of tilapia at decreased cost will continue this upward trend while providing protein for an increasing human population. If the goal of increased aquaculture production is to continue, then an intimate knowledge of GI microbiota and their role in fish health and growth is necessary. The purpose of this review is to highlight information available on the strategies applied to modulate GI microbiota of tilapia and provide suggestions for future research with the ultimate goal of application of these strategies in aquaculture.

Gastrointestinal microbiota in Tilapia

Research on the GI microbiota of aquaculture species has increased in the past two decades as molecular techniques are increasingly refined and accessible (Burr et al., 2005; Sukanta K

Nayak, 2010; Ray, Ghosh, & Ringø, 2012; Vaughan et al., 2000). To fully understand the need for modulation of the intestinal microbiota in tilapia culture, it is essential to review the composition and role of microbiota in growth and health of tilapia. For a review of the intestinal microbiota of fishes other than tilapia, refer to Burr et al. (2005), Cahill (1990), Gómez and Balcázar (2008), Nayak (2010a), Ray et al. (2012), Ringø et al. (2012), and Vadstein et al. (2013).

Colonization and maintenance of the gastrointestinal tract by microbiota

Fishes are host to a variety of microbial species on their gills and skin and throughout their GI tract. Fish are monogastric animals with a complete digestive system comprised of a mouth, pharynx, oesophagus, stomach (in most), small intestine, large intestine, pyloric caeca, liver and pancreas. Microbiota are found throughout the digestive tract; however, due to the high number of species present in the hindgut, it was concluded that these species represented active reproduction in the GI tract and not solely the ingested microflora (Cahill, 1990). A meta-analysis of 25 studies of intestinal microbiota of different fish species revealed that salinity, trophic level, and possibly host phylogeny shape the composition of fish gut bacteria more than any other abiotic and biotic factor examined (Sullam et al., 2012). Del'Duca et al. (2015) determined that the bacteria present in the GI tract of tilapia fingerlings were representative of species found in the water column versus species found in the sediment. This suggests that the microbiota colonizing the intestinal tract are ingested from the water column and settle into the cecum where favourable conditions allow for their continued survival. The composition of the GI microbiota changes rapidly during the transition from the fry stage to the fingerling stage (Cahill, 1990; Del'Duca, Cesar, & Abreu, 2015; Giatsis, Sipkema, Smidt, Verreth, & Verdegem, 2014; Vadstein et al., 2013). Therefore, it is necessary to begin application of diet, antibiotics, probiotics, and prebiotics from the onset of feeding in tilapia larvae to accurately determine the effect of feeds. Giatsis et al. (2014) examined the effects of different aquaculture systems on the colonization of microbiota in the tilapia fingerling intestinal tract and determined that system type (recirculating versus active suspension) accounted for the majority of the variation present in the GI tract of the larvae sampled. This suggests that water quality is the primary indicator of host microbial composition. As reviewed by Nayak (2010a), the stocking densities of tilapia and seasonal rearing conditions also have significant effects on the microbiota present in the GI tract. These are important considerations in experimental design of the investigation of tilapia GI microbiota. For the results to be applicable to the aquaculture industry, rearing conditions should mimic the stocking density and system design of aquaculture farms.

Due to the challenges associated with culturing intestinal microbiota, no detailed information is available on the ecological interactions between species present in the microbiome of the tilapia GI tract (Nayak, 2010). Advances in genetics and molecular biological analyses can begin to answer these questions. For example, DNA microarrays can be used to determine expression of microbial genes in different environments present in the GI tract of fishes (Roh, Abell, Kim, Nam, & Bae, 2010; Vaughan et al., 2000). Additionally, next-generation and third-generation sequencing can be used to rapidly identify different autochthonous microbiota present in fish GI tracts and digital transcriptomics can be used to determine gene expression levels present in samples of diverse microbiota (Ekblom & Galindo, 2011; Roh et al., 2010). For a full review of advances in molecular technology and ecological applications, refer to Ekblom and Galindo (2011), Roh et al. (2010), and Vaughan et al. (2000).

Common intestinal microbiota in tilapia

Early investigations of the intestinal microbiota of tilapia species utilized culture-dependent techniques and were limited to identification of the most common and easily cultured species present. As reviewed by Cahill (1990), the bacteria present in the intestinal tract of tilapia included *Pseudomonas sp.*, *Virbio sp.*, *Aeromonas sp.*, *Enterobacteriaceae sp.*, and other unidentified species. A study by Molinari et al. (2003) examined the microflora in mature tilapia cultured in a semi-intensive system. They found the following bacteria present: *Aeromonas hydrophila, A. veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, Flavimonas oryzihabitans and Plesiomonas shigelloides*. Another study by Pakingking, Palma, & Usero (2015) cultured microbial species present in mature tilapia grown in earthen ponds. They identified the following heterotrophic aerobic bacteria in the tilapia intestinal tract: *Aeromonas hydrophila, A. sobria, Bacillus sp., Citrobacter koseri, Edwardsiella tarda, Edwardisella hoshinae, Enterobacter cloacae, Klebsiella oxytoca, Pasteurella pneumotropica, Photobacterium damselae, Plesiomonas shigelloides, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas luteola, Pseudomonas putida, Shewanella putrefaciens, Staphyloccocus sp., Vibrio cholera, V. fluvialis, V. vulnificus,* and unidentified gram-negative rod species.

Recent advances in the identification of microbiota using molecular techniques have expanded our knowledge of fish microbiota ten-fold, with particular attention given to salmonids (Merrifield & Ringø, 2014; Nayak, 2010). Though many molecular studies summarized below investigate

the modulation of intestinal microbiota of tilapia, to the authors' knowledge, the first attempt to characterize the core microbiota of wild cichlid's GI tract was only recently completed by Baldo, Riera, Tooming-Klunderud, Albà, & Salzburger (2015). Using 16S rRNA pyrosequencing of microbial DNA samples from ten cichlid species, they determined the core bacterial taxa present in at least 80% of the individuals (Table 1.1). The species identified represent a diverse group of phyla including *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Planctomcetes*, and *Verrucomicrobia*. The representative taxon from these phyla included: *Cetobacterium somerae, Clostridium perfringens, Plesiomonas shigelloides, Turicibacter sp., Clostridium XI sp., Aeromonas sp., Neisseriacea, Lachnospiraceae, Clostridiales, Clostridiaceae, Gemmataceae, Acromobacter sp., Bacillus sp.,* and *Pirellulaceae* (Baldo et al., 2015). This study can be used as a baseline for intestinal modulation of microbiota in aquaculture. As discussed in section 2.1, colonization of the intestinal microbiota is highly dependent on the microbiome in the surrounding water; therefore, it is critical to include water quality and microbial analyses in the research of the intestinal microbiota.

Yeast such as *Saccharomyces cerevisiae* are important heterotrophic fermenters in the GI tract and have been proposed as a probiotic for tilapia (Abdel-Tawwab, Abdel-Rahman, & Ismael, 2008; Ayyat, Labib, & Mahmoud, 2014; He et al., 2009; Lara-Flores, Olvera-Novoa, Guzmán-Méndez, & López-Madrid, 2003; D. Merrifield & Ringø, 2014). Several yeast species, including *Kloeckera apiculata, Cadida sp., Metcschnikowia sp.,* and *Rhodotorula sp.*, have been described in finfish other than tilapia (Gatesoupe, 2007; D. Merrifield & Ringø, 2014). Yeasts are known immuno-stimulants in fish and may promote growth and development in older fish (Gatesoupe, 2007). However, despite understanding the importance of yeast in the GI tract of fishes, no studies have investigated the naturally occurring presence or function of eukaryotic autochthonous intestinal microbiota in tilapia.

Effect of microbiota on digestion, growth, and health

The importance of the GI microbiota to host digestion and health were first described in humans and later investigated in commercially important livestock species such as cattle, pig, and chicken (Brisbin, Gong, & Sharif, 2008; Fouhy et al., 2012; Gibson & Roberfroid, 1995; Hooper et al., 2002; Li, Connor, Li, Baldwin Vi, & Sparks, 2012; Richards et al., 2005; Vaughan et al., 2000). In the past three decades, investigations into the GI microbiota of fish have increased. These investigations have highlighted the importance of the host-microbiota interactions in the GI tract for fish growth and health.

Intestinal microbiota break down non-digestible fibres that would otherwise go unutilized by the host and provide nutrients for development and growth of host. By-products of microbe metabolism including enzymes and vitamins can be absorbed along the intestinal tract and further utilized by the host. For endotherms like humans, these microorganisms provide essential enzymes for host survival; however, little information is known on the exact ecological interactions between host and microbiota in ectothermic animals like fish (Merrifield & Ringø, 2014; Nayak, 2010a).

In addition to host growth and development, intestinal microbiota help regulate the host immune system and overall health of the host. Gastrointestinal microbiota provide a number of benefits to host health including: development and maintenance of the mucosal membranes of the host intestinal tract; outcompete pathogenic microorganisms; aid in angiogenesis; and regulate gene expression associated with epithelial proliferation and innate immunity (Merrifield & Ringø, 2014; Nayak, 2010a). With the advance of molecular techniques in recent decades, more work is needed to determine the ecological interactions between members of the GI microbiota and host-microbiota interactions in fish like tilapia. For a full review of the importance of microbiota to fish gastrointestinal tracts, refer to Nayak (2010a).

Microbial pathogens

In addition to beneficial microbiota in the GI tract, there are a number of pathogens competing with the autochthonous bacteria. To date, a number of pathogens invading tilapia species have been described including *Streptococcus sp*. (Amal & Zamri-Saad, 2011) and *Francisella noatunensis subsp. orientalis* (Soto et al., 2013). For a full review of disease in tilapia, refer to textbook “Health Maintenance and Principal Microbial Diseases of Cultured Fishes” by Plumb and Hanson (2011). These microorganisms represent a wide range of virulence and modes of transmission and currently there is no preventative treatment available for all pathogenic microbiota (Plumb & Hanson, 2011). For highly virulent strains that lead to massive die-offs of tilapia, antibiotics are the industry standard for treatment. However, as discussed in section 3, consumer preference is pushing aquaculture facilities to utilize preventative measures and alternatives to antibiotics such as probiotics, prebiotics, and fibrous feed ingredients.

Effects of antibiotics and their alternatives (probiotics, prebiotics, and fibrous feed ingredients) on intestinal microbiota

Antibiotic effects on intestinal microbiota

Antibiotics are natural or synthetic drugs used to kill or inhibit the growth of microorganisms. Their active mechanisms range from cell membrane destruction to inhibition of various metabolic pathways (Defoirdt et al., 2011; Ferguson et al., 2010; L. Welker & Lim, 2011; Martínez Cruz et al., 2012; S. K. Nayak, 2010b; Serrano, 2005; Tuan et al., 2013). For the host, antibiotics have been shown to increase intestinal absorption, digestibility of dietary protein, and potentially stimulate other metabolic processes, though it should be noted that the majority of this evidence stems from research into the porcine intestine and not the fish intestinal tract (Serrano, 2005).

Despite the potential benefits of antibiotic use in aquaculture, there are a number of concerns. Due to rapid asexual reproduction in various pathogens along with the ability for acquired resistance through inter- and intra-species plasmid exchange, there is an emergence of microbial resistance to common antibiotics. Resistant microbiota can wreak havoc on aquaculture facilities while the potential for disease transmission from carcass to humans is concerning to consumers (Defoirdt et al., 2011; Ferguson et al., 2010; L. Welker & Lim, 2011; Martínez Cruz et al., 2012; S. K. Nayak, 2010b; Serrano, 2005; Tuan et al., 2013)).

Another concern is the effect of antibiotics and antibiotic residues on the host. With increasing antibiotic dosage, there is the potential for increased bioaccumulation of antibiotic residues in fish tissues that may later be consumed by humans or other agriculturally important species. Depending on the antibiotic, it may act as a mutagen, allergen, toxin, or have no effect on the consumer (Serrano, 2005). Additionally, antibiotic consumption will negatively affect the host's intestinal microbiota. As described in Section 2, intestinal microbiota are essential to mammalian growth, nutrient utilization, and health. Given the potential for negative effects on the consumer, antibiotic accumulation and potential impacts on consumers are increasingly tested and regulated. Despite these regulations, consumers are increasingly interested in "antibiotic-free" products and the market is moving away from the overuse of antibiotics that were prevalent in early aquaculture production (Defoirdt et al., 2011; Ferguson et al., 2010; Martínez Cruz et al., 2012; Nayak, 2010b; Serrano, 2005; Tuan et al., 2013; Welker & Lim, 2011).

Antibiotic Alternatives

A number of alternatives to antibiotics proposed for tilapia and a complete review of the available alternatives to antibiotics in aquaculture is provided by Defoirdt et al. (2011). This paper reviews those antibiotics and alternatives whose effects on the intestinal microbiota of tilapia were examined in detail (Table 1.2). For more information on the development of alternatives to antibiotics in aquaculture, refer to Defoirdt et al. (2011) and the textbook "Aquaculture Nutrition: Gut Health, Probiotics, and Prebiotics" edited by Merrifield and Ringø (2014).

As far as the authors are aware, the first attempt to identify the effects of antibiotics on the autochthonous intestinal microbiota of tilapia using culture-independent techniques was done by Zhou et al. (2009b). They examined the effect of feeding Potassium diformate (KDF) to hybrid tilapia (*O. niloticus* × *O. aureus)* along with three antibiotic treatments: flavomycin (8 mg/kg), quinocetone (100 mg/kg), and flavomycin (4 mg/kg) + quinocetone (50 mg/kg) over an eight-week trial. The results suggested that KDF addition had no significant effect on tilapia growth performance, feed conversion ratio or survival compared to the control, but KDF treatments of 3.0 and 6.0 g/kg had increased growth performance and feed conversion ratio compared to the flavomycin + quinocetone. There were changes in the GI microbiota of tilapia with increases in some species and decreases in others, which need to be investigated further in subsequent studies. A similar study by He et al. (2010) investigated the effects of the antibiotic growth promoters flavomycin and florfenicol on the autochthonous intestinal microbiota of juvenile hybrid tilapia (*O. niloticus* × *O. aureus)*. They also determined that the application of antibiotics significantly decreased autochthonous bacterial diversity, with the effects of florfenicol overshadowing flavomycin. This research confirmed results from culture-dependent studies on the effects of antibiotics on GI microbiota and paved the way for systematic testing of common antibiotics and antibiotic alternatives in aquaculture.

Building off this work, subsequent studies investigated the effects of: antibiotics in sequence with prebiotics such as DVAQUA® and subsequently challenged with *Aeromonas hydrophila* (Zhou et al., 2011); the immunostimulant Ergosan® dietary alginic acid (Merrifield et al., 2011); and the antibiotic florfenicol in combination with the amino acid derivative betaine (He et al., 2012). The results from these studies were mixed: the DVAQUA® prebiotic failed to recuperate the intestinal microbiota of fish in the antibiotic treatment (Zhou et al., 2011); the dietary alginic

acid did not adversely impact the indigenous intestinal microbial balance and did not impact the epithelial brush border integrity (Merrifield et al., 2011); and florfenicol's inhibitory effect overshadowed the beneficial effects of betaine (He et al., 2012). As Nayak (2010a) suggested, the GI microbial ecosystem dynamics are exceptionally complicated and more work needs to be done to understand the composition of the GI microbial species present, their colonization dynamics, and the interspecies interactions on one another and their host.

To the authors' knowledge, these are the only five studies so far which evaluated the effects of antibiotics on the intestinal microbiota of tilapia using culture-independent techniques. Another study investigated the effect of organic acids blend and oxytetracycline on the body mass growth, nutrient utilization, and total cultivable gut microbiota of the red hybrid tilapia and its resistance to *Streptococcus agalactiae* (Koh, Romano, Zahrah, & Ng, 2014). Their twenty-week trial tested three diets; 0.5% organic acids blend, 1.0% organic acids blend, and 0.5% oxytetracycline against the control diet with no additives. While oxytetracycline and the organic acids blend diets performed similarly in growth, nutrient utilization, and survival, both of the organic acids blend diets had significantly lower total faecal bacterial culture counts (Koh et al., 2014). This study confirmed the effect of antibiotics on the GI microbiota of tilapia; however, they used culture-dependent techniques and thus cannot report the effect of organic acids blend and oxytetracycline on the facultative anaerobes in the tilapia intestine. Future studies should examine culture-independent techniques to fully understand the ecological interactions between species in the GI tract and the effects of antibiotics and antibiotic alternatives on the entire GI microbial community.

It is known that antibiotics decrease the GI microbiota of the host and these studies attempt to highlight the specific bacterial communities affected by antibiotics, with varied results. With the increase in disease-resistant microorganisms due to the overuse of antibiotics in commercial aquaculture, it would be beneficial to determine how antibiotic alternatives affect the intestinal microbiota before their wide-scale application is considered in commercial facilities.

Probiotics

The vast majority of research into the intestinal microbiota of tilapia focused on the use of probiotics and their ability to survive within and benefit their host (Table 1.2). For a formal review of the probiotic research in aquaculture, refer to the following: Burr et al. (2005), Dimitroglou et al. (2011), Gómez and Balcázar (2008), Welker and Lim (2011), Martínez Cruz et al. (2012),

Nayak (2010b), and Tuan et al. (2013). To date, the following probiotics have been tested for tilapia: *Bacillus sp.* (Aly, Mohamed, & John, 2008; Apún-Molina, Santamaría- Miranda, Luna-González, Martínez-Díaz, & Rojas-Contreras, 2009; Del'Duca, Cesar, Diniz, & Abreu, 2013; He et al., 2013), *Bifidobacterium bifidum* (Ayyat et al., 2014), Biogen® (El-Haroun, Goda, & Kabir Chowdhury, 2006), *Chlorogloeopsis sp.* (Merrifield et al., 2010), *Lactobacillus sp.* (Ayyat et al., 2014; Jatobá et al., 2011; Liu et al., 2013; Pirarat et al., 2011), *Micrococcus luteus* (Abd El-Rhman, Khattab, & Shalaby, 2009)*, Pediococcus acidilacici* (Ferguson et al., 2010; Standen et al., 2013), *Pseudomonas sp.* (Abd El-Rhman et al., 2009), *Saccharomyces cerevisiae* (Abdel-Tawwab et al., 2008; Ayyat et al., 2014; He et al., 2009; Lara-Flores et al., 2003; Zhou, He, et al., 2009), and *Streptococcus sp.* (Ayyat et al., 2014; Lara-Flores et al., 2003). All of the studies found some positive effects of the probiotics on growth, disease resistance, intestinal microbiota, and/or increased immune parameters. These results support prior studies on the importance of probiotics on intestinal microbiota, host immune response, and overall health of the host (Martínez Cruz et al., 2012; Sukanta K Nayak, 2010; Tuan et al., 2013).

While the goal is to develop probiotics that are beneficial for tilapia production and health, there are vast differences in the methodologies. For example, when testing the potential of *Saccharomyces cerevisiae* on growth and health of tilapia fry, Abdel-Tawwab et al. (2008) chose to measure body mass growth parameters, histology, blood chemistry, bacterial colony counts using the Miles-Misra technique, and challenged the tilapia after the twelve-week trial with *Aeromonas hydrophila* by direct injection (Abdel-Tawwab et al., 2008). He et al. (2009) chose to investigate only the fermentation product DVAQUA® of *Saccharomyces cerevisiae* on juvenile hybrid tilapia over an eight week period and measured the growth of tilapia, catalogued the autochthonous bacteria by isolating V3 region of 16S rDNA using PCR-DGGE and sequencing, measured lysozyme activity, C3 and C4 serum levels, phagocytic activity index, and respiratory burst activity (He et al., 2009). While both studies conclude that *Saccharomyces cerevisiae* and its fermentation products are beneficial to tilapia growth and intestinal health, the optimal levels vary depending on the variables of interest.

In addition, Ayyat et al. (2014) tested the effects of *Saccharomyces cerevisiae* in combination with *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Bifidobacterium bifidum* on tilapia fingerlings. They analysed growth performance, feed efficiency, survival rate, blood protein, albumin, globulin, and plasma enzymes along with an economic analysis of the viability of each of the different treatments over the fourteen-week trial. *Lactobacillus acidophilus,*

Bifidobacterium bifiduim, and the mixed probiotic groups had higher survival in *A. hydrophila* challenge. Feed cost, return on weight gain, and profit margin increased in the highest variety probiotic group (Ayyat et al., 2014). This is the only economic analysis the authors are aware of for probiotics in tilapia to date. These three trials highlight the variety of trial designs and analyses for probiotics in tilapia aquaculture and the need for standardization of the techniques to cross-compare probiotic efficacy. In addition to standardizing techniques, it is important to study the timing of probiotic treatments, effects of probiotics on water quality and the environment, and potential pathogenic mutations of probiotic strains (Tuan et al., 2013).

Prebiotics

The use of prebiotics in aquaculture is an emerging field, with most research occurring over the past decade. For a full review of prebiotic use in aquaculture, refer to Burr et al. (2005) and Ringø et al. (2010). To date, the following prebiotics have been investigated for their effects on the gastrointestinal microbiota of tilapia: dietary yeast culture and short-chain fructo-oligosaccharides (Zhou, et al., 2009), inulin and Jerusalem artichoke (Tiengtam, Khempaka, Paengkoum, & Boonanuntanasarn, 2015), propolis (Abd-El-Rhman, 2009), and vitamin C and inulin (Ibrahem, Fathi, Mesalhy, & Abd El-Aty, 2010). All four prebiotics increased the growth, immune response, and microbial diversity of the intestinal tract of tilapia (for those tested), confirming their beneficial effects on the overall health of the organism. To further explore their potential use in aquaculture, future experiments should consider the effects of prebiotics in conjunction with probiotics, antibiotics, and alternative feed ingredients modelled after He et al. (2012).

Alternative feed ingredients and feed additives

Research into alternative feed ingredients for tilapia aquaculture has primarily focused on protein, the main cost of feed production. Several reviews of alternative protein sources for tilapia have been published and the authors refer to these works for a complete description of the alternative feeds in tilapia: El-Sayed (1999), Kuhn et al. (2009), and Nguyen (2008). For the purpose of this review, the authors will focus on alternative feed ingredients and feed additives and their effects on intestinal microbiota of tilapia.

The following dietary supplements and alternative feed ingredients have been investigated to determine their effect on intestinal microbiota of tilapia: the dietary supplement and phytobiotic Sangrovit® (Rawling, Merrifield, & Davies, 2009); dietary *Chlorogloeopsis* (Daniel Lee Merrifield

et al., 2010); and the dietary supplement NovaSil (Zychowski et al., 2013). Of these supplements, only Sangrovit® had significant effects on the growth rate and weight gain of tilapia, but no significant differences were present in the culture-dependent microbiota identified. Both Merrifield, et al. (2010) and Zychowski, et al. (2013) examined used PCR-DGGE to examine the microbial diversity in the intestinal tract of tilapia in their trials. They reported that there were no significant differences between treatments and the control, suggesting that *Chlorogloeopsis* and NovaSil® do not have an effect on the intestinal microbiota; however, both suggest more investigation into the microbial ecology of tilapia intestine is necessary.

Recently, Pedrotti et al., (2015) examined the effect of dietary carbohydrates dextrin, ground corn, wheat, cassava bagasse, and broken rice on the intestinal microbiota of *O. niloticus* and *Rhamdia quelen* using PCR-DGGE and sequencing of the distal intestine based on their first experimental results, which indicated that more amylolytic bacteria were present in this region. They determined that the diets altered the composition of the bacterial populations present in the intestine of fish and the changes were also dependent on the species of fish (Pedrotti et al., 2015). This is an important first step in the investigation of the effect of feed ingredients on the intestinal microbiota of tilapia and should be used as a model for optimizing diets containing alternative feed ingredients.

Another study by Leenhouwers et al. (2008) used inocula from *O. niloticus* to study the *in vitro* fermentability of glucose, native wheat starch, arabinoxylan and whole wheat. They determined that fermentation rates were highest for glucose and lowest for whole wheat. There were vast differences in fermentability and composition of fermentation end-products between the carbohydrate sources (Leenhouwers et al., 2008). No attempt was made to culture or otherwise identify the microorganisms in the tilapia inoculum. This study focused on wheat and wheat-based products as this is the primary component of commercial fish diets; however, this study could be applied to alternative carbohydrate sources and their effects on intestinal microbiota using molecular culture-independent techniques such as PCR-DGGE and next-generation or third-generation sequencing described below.

Perspectives and future research considerations

Over the past two decades, research into the intestinal microbiota of tilapia increased with the goal of determining optimal levels of antibiotics, alternative to antibiotics like probiotics, prebiotics, and alternative feed ingredients for tilapia diets. The research highlighted above is an

443 important starting point for the determination of optimal levels of antibiotics, probiotics,
444 prebiotics, and phytobiotics along with the effects of alternative feeds on the intestinal
445 microbiota of tilapia. However, there is still a lot of research required before a feeding regime
446 can be designed to promote the growth, health, and quality of tilapia being raised for human
447 consumption.
448
449 *Experimental design considerations*
450 The studies covered in this review vary in species and age of the tilapia, length of the study,
451 species of bacteria used as challenge organism, and culture dependent versus independent
452 methods, making cross-comparisons of their results exceedingly difficult. To optimize the
453 intestinal microbiota of tilapia to promote growth, health, and quality of tilapia, the following
454 experimental design parameters should be taken into consideration: age, research system,
455 seasonal effects, and biotechnology techniques/applications.
456
457 The first consideration is the age of tilapia species used in the study. Tilapia larvae and fry
458 represent the colonization period for intestinal microbiota and thus are the optimal age for
459 investigating alterations to the intestinal microbiota as preventative measure (de Blas et al.,
460 2010; Giatsis et al., 2014). If alternative to antibiotics like probiotics, and prebiotics are
461 considered as a treatment rather than a preventative measure, it may be beneficial to use older
462 tilapia.
463
464 Giatsis et al. (2014) investigated the colonization dynamics of intestinal microbiota in *O. niloticus*
465 larvae and determined that the type of system used in research (recirculating vs. active
466 suspension) also had a significant effect on the intestinal microbiota in the larvae. Del'Duca et
467 al. (2015) determined that water quality also had a significant effect on the colonization of
468 intestinal microbiota. Therefore, it would be beneficial to monitor the environmental microbiota in
469 addition to the intestinal microbiota and compare between tanks/treatments and other test
470 variables.
471
472 In aquaculture settings, the fish are subjected to varying temperatures associated with the
473 season, stocking densities, and water quality. However, there is little information on the effects
474 of ecological and environmental factors on the intestinal microbiota of fishes (Wong & Rawls,
475 2012). These effects should be investigated in more detail to determine their impact on intestinal

476 microbiota before determining the effects of antibiotics, prebiotics, probiotics, and alternative
477 feeds.
478
479 Finally, with the development of biotechnology applications such as microarrays and third-
480 generation sequencing, further investigation into the identification and microbial ecology of the
481 fish intestinal tract should be the priority. Investigation into the complex interactions within the
482 microbiome can lead to a better understanding of the interactions between intestinal microbiota
483 and their hosts. Once these interactions are understood, then the work into the modulation of
484 the intestinal microbiota to benefit the host can progress.
485
486 *Alternative feed ingredients*
487 A number of alternative feed ingredients have been evaluated for their use in tilapia aquaculture
488 (A.-F. M. El-Sayed, 1999; Ng & Romano, 2013; Tri Niu Nguyen, 2008). The majority of these
489 feed ingredients have been proposed as alternative protein sources to reduce costs and
490 promote growth of tilapia. However, very few of these studies investigated the effects on
491 intestinal microbiota. While carbohydrate sources are the primary modulator for intestinal
492 microbiota, the shift from fishmeal to plant-based protein sources will also alter the carbohydrate
493 composition of the diet and should be further investigated. Additionally, co-products and
494 alternative feed ingredients rich in fibre may act as prebiotics stimulating the growth of beneficial
495 intestinal microbiota. Due to their herbivorous nature, tilapia are capable of consuming diets with
496 a carbohydrate content ranging from 30-40% (Mjoun Kamal, Kurt.A, & Brown Michael L., 2010).
497 It is therefore surprising that very few of the research papers investigating the effects of
498 proposed alternative feed ingredients have not included an intestinal microbiota analysis.
499
500 *Economic considerations*
501 One of the main concerns in aquaculture research is the production of high-quality products at
502 the lowest possible cost. The vast majority of studies into the growth and health of tilapia,
503 particularly studies on alternative feed ingredients, cited economic concerns as one of the
504 driving factors for their work; however, relatively few of these studies reported economic
505 analyses within their results. If the driving factor of the research is to reduce costs through the
506 promotion of tilapia growth, health, etc., then at least a basic economic analysis should be
507 included.
508
509

Conclusions

The complex interactions in the microbiome of the tilapia gastrointestinal tract merits further research, particularly concerning the modulation of the microbiota in order to promote the health and growth of captive fish. The use of probiotics, prebiotics, and alternative feed ingredients has the potential to positively affect growth, intestinal health, nutrient digestibility, water quality, and reproduction in aquaculture and agriculture species. Special consideration should be taken in the experimental design of such applications and quantification of the microbiota in order to ensure the results are replicable in large-scale aquaculture facilities. Additionally, economic models should be applied when investigating the use of probiotics, prebiotics, and alternative feed ingredients in aquaculture. The cost-benefit analyses will provide additional criteria for commercial farms to evaluate the potential use of antibiotic alternatives in their facilities

Table 1.1 Cichlid core bacterial taxa, defined by presence in at least 80% of the individuals (i.e. 20/25, excluding AstburLAB), a minimum of one representative per species and consistently in both 16S libraries (Reproduced from Baldo et al., 2015).

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Phylum	Class	Order	Family	Genus	Species
Actinobacteria	Actinobacteria	Actinomycetales	Aeromonadacea	Cetobacterium	*Cetobacterium somerae*
Bacteroidetes	Alphaproteobacteria	Bacillales	Clostridiaceae	Clostridium	*Clostridium perfringens*
Firmicutes	Bacilli	Bacteroidales	Enterocateriaceae	Plesiomonas	*Plesiomonas shigelloides*
Fusobacteria	Bacteroidia	Burkholderiales	Fusobacteriaceae	Turicibacter	
Plactomycetes	Betaproteobacteria	Clostridiales	Lachnospiraceae		
Proteobacteria	Clostridia	Fusobacteriales	Neisseriaceae		
Verrucomicrobia	Fusobacteria	Turicibacterales	Pirellulaceae		
	Gammaproteobacteria		Rhodobacteraceae		
	Plactomycetia		Turcibacteraceae		

527 **Table 1.2** Summary of microbial analysis of dietary supplements on tilapia intestinal microbiota.

Species	Analysis	Results	Source
Oreochromis niloticus	Effect of *Micrococcus luteus* and *Pseudomonas* species isolated from the gonads and intestine of Nile tilapia on growth performance, survival rate, blood parameters and chemistry	Best growth rate, feed utilization, and survival rate in diet with *M. luteus*	*Abd El-Rhman et al., 2009*
Oreochromis niloticus	Effect of propolis on the growth rate, feed conversion, blood cell counts, and challenge by *Aeromonas hydrophila*	Propolis-ethanolic-extract enhanced growth, immunity, and resistance compared to crude propolis	*Abd-El-Rhman, 2009*
Oreochromis niloticus	Evaluate *Saccharomyces cerevisiae* as growth and immunity promoter in fry when challenged with *Aeromonas hydrophila*	Final weight, weight gain, and specific growth rate increased significantly with increased yeast. Survival increased with yeast after injection of *A. hydrophila*. Lowest bacterial count in yeast samples vs. control.	*Abdel-Tawwab et al., 2008*
Oreochromis niloticus	Evaluate *Lactobacillus acidophilus, Streptococcus thermophiles, Bifidobacterium bifidu, and Saccharomyces cerevisiae* as a combination probiotic in the diet of fingerlings using growth rate, food consumption, and feed conversion ratios	Best growth rate, food consumption, and feed conversion seen in group fed probiotic "cocktail." Feed cost, return on weight gain, and profit margin increased in probiotic cocktail group. *Lactobacillus acidophilus, Bifidobacterium bifiduim*, and cocktail groups had higher survival in *A. hydrophila* challenge	*Ayyat et al., 2014*

Oreochromis niloticus	Isolated bacteria from tilapia, sediment, and water. Determined potential probiotics in vitro and fed strains to tilapia. FISH identification of probiotics in GI tract of tilapia	*Bacillus* showed most potential as probiotic in tilapia diet	*Del'Duca et al., 2013*
Oreochromis niloticus	Effect of dietary probiotic Biogen on tilapia. Measured weight gain, SGR, protein efficiency ratio, protein productive value, energy retention	All parameters significantly higher in diets containing probiotic than control Cost-benefit analysis suggested probiotic was beneficial	*El-Haroun et al., 2006*
Oreochromis niloticus	Effect of *Pediococcus acidilactici* PCR-DGGE of 16S rDNA, histology of gut and blood, growth performance and survival	Direct antagonism of gastric *Pediococcus acidilactici*, gut histology unaffected, blood leucocyte levels and serum lysozyme activity elevated, no change to growth, improved survival with *Ped. Acidilactici*	*Ferguson et al., 2010*
Oreochromis niloticus × *Oreochromis aureus*	Effects of dietary saccharomyces cerevisiae fermentation product on growth performance, intestinal autochthonous bacterial community using DGGE 16S rDNA V3 region and non-specific immunity	No significant effect on growth, feed conversion, or survival. Variation may be due to seasonal changes. Enhanced nonspecific immunity	*He et al., 2009*
Oreochromis niloticus ×	Effects of the antibiotic growth promoters flavomycin and florfenicol on the	Flavomycin significantly altered intestinal microbiota Florfenicol reduced the # of autochthonous bacteria and overwhelmed effects of flavomycin in diet	*He et al., 2010*

Oreochromis aureus	autochthonous intestinal microbiota using 16S rDNA V3 region and real-time PCR		
Oreochromis niloticus × *Oreochromis aureus*	Effects of betaine and the antibiotic florfenicol on the autochthonous bacteria using 16S rDNA V3 region and quantitative PCR	Betaine can promote some intestinal autochthonous bacteria, and florfenicol play a depressor role. When combined together, florfenicol may overshadow the effect of betaine on the predominant intestinal bacteria of tilapia	*He et al., 2012*
Oreochromis niloticus × *Oreochromis aureus*	Effects of low doses of dietary Bacillus subtilis C-3102 on the production, intestinal cytokine expression and adhesive bacteria using 16S rRNA V3 region and real-time PCR	B. subtilis C-3102 altered the autochthonous gut bacterial communities, significantly increased the total amounts of adhesive viable bacteria, induced upregulation of intestinal cytokine expression (IL-1b, TGF-β and TNF-α) and downregulation of intestinal HSP70.	*He et al., 2013*
Oreochromis niloticus	Effect of inulin and Ascorbic acid on improving the performance as well as the immunity of Nile tilapia challenged with A. hydrophila	Vitamin C at dose rate of 500 mg for one month could be a potential dietary supplement in place of inulin.	*Ibrahem et al., 2010*
Oreochromis niloticus	Effect of diet supplemented with probiotic for Nile tilapia in polyculture system with marine shrimp using culture-dependent methods	Viable heterotrophic bacterial counts decreased in presence of lactobacillus plantarum	*Jatobá et al., 2011*

Oreochromis niloticus × *Oreochromis aureus* hybrids	Effect of organic acids blend and oxytetracycline on growth, nutrient utilization, total cultivable gut microbiota, and resistance to *Streptococcus agalactiae*	Similar growth and resistance to *S. agalactiae*, but significantly lower colony forming units of adherent gut bacteria in fish fed organic acids blend diet.	*Koh et al., 2014*
Oreochromis niloticus	Effect of *Streptococcus faecium Lactobacillus acidophilus,* and *Saccharomyces cerevisiae* on growth performance on fry over 9 weeks at various stressors – low protein and high stocking density	The diet containing 40% protein and yeast had best growth performance and feed efficiency	*Lara-Flores et al., 2003*
Oreochromis niloticus and *Dicentrarcus labrax*	Investigated in vitro fermentability of glucose (GL), native wheat starch (WS), arabinoxylan (ABX) and whole wheat (WHT) using inocula of Nile tilapia and European sea bass. Cumulative gas production was measured for 168 h. At the end of incubation, fermentation end-products were measured.	Intestinal microbes from Nile tilapia and European sea bass have the potential to ferment carbohydrates. Large differences exist in fermentability and composition of fermentation end products between carbohydrates. (No microbiota identification)	*Leenhouwers et al., 2007*
Oreochromis niloticus × *Oreochromis aureus* hybrids	Compare the effects of two *Lactobacillus* strains on survival and growth, adhesive gut bacterial communities, immunity, and protection against pathogenic bacteria	No significant differences in survival rate, weight gain, or feed conversion. Highly adhesive *Lactobacillus brevis* showed rapid response in	*Liu et al., 2013*

	(*Aeromonas hydrophila*) using PCR DGGE of 16S rrs DNA and sequencing	intestinal microbiota and appeared to protect against toxic effects of *A. hydrophila.*	
Oreochromis niloticus	Assessment of chlorogloeopsis as a novel microbial dietary supplement using light and scanning electron microscopy for intestinal morphology PCR-DGGE 16S rRNA V3 region	Highly similar microbial communities – richness and species diversity Autochthonous less diverse, dense, etc. than allochthonous bacteria	*Merrifield et al., 2010*
Oreochromis niloticus	Effect of dietary alginic acid on juvenile tilapia intestinal histology and growth performance using Light and scanning electron microscopy for intestinal morphology and PCR-DGGE 16S rRNA V3 region	Highly similar microbial communities – richness and species diversity Non-significant elevated survival and protein content seen in aglinic acid group	*Merrifield et al., 2011*
Oreochromis niloticus	The effect of dietary carbohydrates on the autochthonous microbiota using PCR DGGE 16S rRNA V3 region and sequencing and culture-dependent methods	No difference in autochthonous levels among carbohydrate sources within species. Jundia on broken rice had higher culturable bacteria. Very few species IDs	*Pedrotti et al., 2015*
Oreochromis niloticus	Modulation of intestinal morphology by *Lactobacillus rhamnosus* GG	All parameters significantly higher in diet containing *L. rhamnosus* suggesting its value as a probiotic	Pirarat et al., 2011

Oreochromis niloticus	Preliminary assessment of dietary supplementation of Sangrovit® on growth performance and health using culture-dependent methods	Sangrovit significantly increased the final body weight, weight gain, mean daily feed intake, and specific growth rate. No significant difference in microbes	*Rawling et al., 2009*
Oreochromis niloticus	Evaluate the probiotic effect of Pediococcus acidilactici on Nile tilapia (Oreochromis niloticus) intestinal health, probiotic levels, and system level responses using light microscopy and real-time PCR	The probiotic has a protective action on the intestinal mucosal cells. These immunological modulations did not impair growth performance or the remaining haematological and zootechnical parameters	*Standen et al., 2013*
Oreochromis niloticus	Effects of inulin and Jerusalem artichoke (*Helianthus tuberosus*) as a prebiotic in juveniles using growth performance, blood chemistry, immune assay, and histology	Both inulin and artichoke had significantly higher growth performance, red blood cell number, increased blood chemistry, improved immune activity. Both have potential as prebiotics in fish feed.	*Tiengtam et al., 2015*
Oreochromis niloticus × *Oreochromis aureus* hybrids	Effects of dietary potassium diformate (KDF) on growth performance, feed conversion and intestinal bacteria using 16S rDNA PCR DGGE	No significant effects on growth performance, feed conversion, or survival. However, KDF3 and KDF6 better GP, FCR Positive effects on gut microbiota in KDF3 and KDF6	*Zhou et al., 2009a*
Oreochromis niloticus ×	Effects of dietary yeast culture (YC) or short-chain fructo-oligosaccharides (FOS) on intestinal autochthonous bacterial	Obvious effects of dietary prebiotics on intestinal communities. Higher variation detected within the dietary YC group, possibly due to the effects of	*Zhou et al., 2009b*

Oreochromis aureus hybrids	communities in juveniles using 16S rDNA denaturing gradient gel electrophoresis (DGGE)	certain immune-stimulating agents in YC on the immunity response of hybrid tilapia.	
Oreochromis niloticus × *Oreochromis aureus* hybrids	Investigate whether dietary antibiotic-induced changes in the fish intestinal microbiota altered host physiological responses to the infection with *Aeromonas hydrophila* using 16S rDNA denaturing gradient gel electrophoresis (DGGE)	Infection with *A. hydrophila* reduced the gut bacterial evenness, and slightly improved the gut bacterial richness in antibiotic-supplemented tilapia. *A. hydrophila* infection affected non-specific immunity such as serum lysozyme activity and serum alternative complement pathway (C3 and C4) activities regardless of hybrid tilapia fed antibiotic-supplemented diets.	*Zhou et al., 2011*
Oreochromis niloticus	Ability of NovaSil (NS) clay to sorb and mitigate the toxic effects of aflatoxin B1 (AFB1) by monitoring growth performance, targeted innate immunological function, intestinal microbial community, and histology.	Aflatoxin significantly decreased weight gain, feed efficiency, hepatosomatic index and macrophage extracellular superoxide anion production in tilapia, regardless of NS addition to the diet. The overall results regarding the efficacy of NS were mixed. No significant differences were found among treatment groups for microbial community dynamics.	*Zychowski et al., 2013*

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CHAPTER 2 EVALUATION OF MORINGA STENOPETALA LEAVES AS A PREBIOTIC IN HYBRID TILAPIA PRODUCTION

Abstract

Prebiotics have increasingly been used in aquaculture to promote health. Moringa (*Moringa stenopetala*) is an East-African tree used primarily for food but is known to contain medicinal benefits. Moringa leaves are rich in fiber, thus they may serve as a prebiotic. This study investigated the role of moringa leaves as a prebiotic in hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) production. Four hundred tilapia fingerlings (7.1 ± 0.55g initial body weight) were divided between 16 tanks and fed with one of the four diets with the following inclusion levels of moringa leaves: 0% (control), 6%, 9%, or 12% for 84 days. The specific growth rate, feed conversion ratio, protein efficiency ratio, and percent survival were recorded over the course of the study. Intestinal samples were collected monthly and subjected to microbiota characterization using PCR-DGGE and metagenomic sequencing. The results indicate that inclusion of moringa leaves altered the intestinal microbiota of tilapia, particularly the increase in Caulobacteraceae, Oxalobacteraceae, and Comamonadaceae, suggesting that it may be used as a prebiotic in tilapia aquaculture. Additionally, the reduction in proteobacterial levels suggests that *M. stenopetala* may have anti-inflammatory properties, similar to those recorded in *M. oleifera*.

Keywords: Prebiotics, Moringa, Microbiota, Intestinal Microbiome

Introduction

The intestinal microbiome is a complex set of interactions between microbes and their host. The mutualistic relationship between the host and its intestinal microbiota determines the overall health, and thus the evolutionary fitness of the host. Microbes help regulate the digestive processes, immune system, and even brain function of the host; therefore, understanding their function and modes of action are critically important to promoting the health and well-being of humans and food animals through the use of prebiotics (Fouhy, Ross, Fitzgerald, Stanton, & Cotter, 2012; Giatsis, Sipkema, Smidt, Verreth, & Verdegem, 2014; Gibson & Roberfroid, 1995; Haygood & Jha, 2018; Hooper, Midtvedt, & Gordon, 2002; Jha & Berrocoso, 2016; Llewellyn, Boutin, Hoseinifar, & Derome, 2014; Nicholson et al., 2012; Round & Mazmanian, 2009).

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already residing in the colon, and thus attempt to improve host health (Gibson & Roberfroid, 1995). A number of prebiotics have been investigated for human consumption (Gibson & Roberfroid, 1995; Reid et al., 2003; Roberfroid, 2007) and food animal consumption (Burr et al., 2005; Haygood & Jha, 2018; Patterson & Burkholder, 2003; Ringø et al., 2010) to promote their overall health. While *Moringa oleifera* has been studied for its anti-cancer (Gopalakrishnan, Doriya, & Santhosh Kumar, 2016; Kooltheat et al., 2014; Sreelatha, Jeyachitra, & Padma, 2011; Tiloke, Phulukdaree, & Chuturgoon, 2013; Vasanth, Ilango, MohanKumar, Agrawal, & Dubey, 2014), anti-inflammatory (Kooltheat et al., 2014), and water purification properties (Desa, 2010), no work has been done on *M. stenopetala* in this regard. *Moringa stenopetala* is easily cultivated in tropical and subtropical climates, even in marginal conditions. It is currently consumed as an important dry season vegetable and used as alternative medicine in different parts of the world (Bennett et al., 2003). Moringa leaves are high in crude fiber, crude protein, calcium, vitamin C, β-carotene, and produce secondary metabolites like flavonoids that are beneficial for the host intestinal tract (Gopalakrishnan et al., 2016; Imungi et al., 2011; Melesse, Bulang, & Kluth, 2009; Richter, Siddhuraju, & Becker, 2003). Crude fiber and vitamin C have been shown to modulate the intestinal microbiota in humans and food animals (Ibrahem, Fathi, Mesalhy, & Abd El-Aty, 2010; Jha & Berrocoso, 2016; Queiroz-Monici, Costa, Da Silva, Reis, & De Oliveira, 2005). It has been shown in humans and swine that inclusion of dietary fiber and reduction of crude protein in diets may counteract the negative effects of protein fermentation in the gut by reducing ammonia concentration and shifting nitrogen excretion pathways in the gut (Gibson & Roberfroid, 1995; Jha & Leterme, 2012; Jha & Berrocoso, 2016). These factors make Moringa leaves a potential prebiotic for human and food animals.

The purpose of this study was to evaluate the effect of dietary inclusion of *Moringa stenopetala* leaves on the intestinal microbiome using tilapia as a host species. Our hypothesis was that the incorporation of moringa leaves in tilapia diets will significantly alter the intestinal microbial composition.

Materials and methods

All animal procedures were conducted in accordance with the approval (protocol #13-1639) from the Institutional Animal Care and Use Committee (IACUC) of the University of Hawaii, Honolulu, HI, USA.

Feeding trial

Four hundred fingerling tilapia (*Oreochromis niloticus* x *O. mossambicus*) with an initial weight of 7.1 ± 0.55g were randomly and equally allocated to one of four treatments with four replicate freshwater tanks each (experimental unit). Four moringa leaves diets (inclusion levels: 0, 6, 9, and 12%) were offered twice daily up to 4 percent body weight daily. Water quality parameters (pH, nitrates, nitrites, ammonia, temperature, and dissolved oxygen, DO) were monitored weekly to ensure they were within the optimal range for tilapia. The DO concentration, temperature, and pH were measured routinely using the HQ40d Portable Water Quality Lab Package (Hach, Loveland, CO, USA). Total ammonia nitrogen (TAN, NH_3-N) was measured using the reaction kit Ammonia TNTplus, ULR (TNT 830, Hach, Loveland, CO, USA). Nitrite-N (NO_3-N) was measured using the reaction kit Nitrite TNTplus, LR (TNT 839, Hach, Loveland, CO, USA). Nitrate-N (NO_2-N) was measured using the reaction kit Nitrate TNTplus, HR (TNT 836, Hach, Loveland, CO, USA). Water temperature was maintained at 22.5±0.8ºC, DO was maintained at 10.7±0.7 mg/L, pH was 7.9±0.25, NH_3-N was 3.4±3.6 ppm, NO_3-N was 2.97±2.0 ppm, and NO_2-N was 12.4±22.7 ppm. A 12 h light／12 h dark photoperiod was maintained throughout the animal study period.

Experimental diet

Moringa stenopetala were harvested from the University of Hawaii Waimanalo Research Station (Waimanalo, HI, USA). The sample was ground to pass through a 1.0 mm-mesh screen using a laboratory mill and dried in an air-dry oven. The ground sample was subjected to proximate analysis according to the Association of Official Analytical Chemists standard procedures (AOAC, 2007) with specific methods as follows: DM (135°C for 2 h, AOAC 930.15); Crude protein (CP) analysis (AOAC 976.05) by determining nitrogen (N) using LECO TruSpec CN analyzer (LECO Corp., St. Joseph, MI, USA) and N multiplied by 6.25 to get the % CP; crude fiber (AOAC 978.10), crude fat (AOAC 920.39; using Soxhlet apparatus and petroleum ether), and ash (AOAC 942.05). Vitamin C analysis was performed on the moringa leaf samples (AOAC 967.22). Four isocaloric and isonitrogenous diets were formulated to meet the nutritional requirements of tilapia fry (Mjoun Kamal et al., 2010). All diets were analyzed following the same methods as moringa leaf samples (described above); the ingredient composition and analyzed nutritional values of all diets are presented in Table 2.1.

Intestinal microbiota and environmental microbiota sampling

Tilapia intestinal microbiota was sampled monthly and the intestinal samples from two fish per tank were pooled (four samples per treatment). The fishes were euthanized with 0.6 g l^{-1} Tricaine Methanesulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA), buffered with 0.12 g l^{-1} sodium bicarbonate in water originating from the corresponding rearing tank. Subsequently, fish were rinsed with 70% ethanol and sterile water before dissecting out aseptically the gut under a dissection microscope. Whole gut samples were flash frozen in liquid nitrogen and stored individually at -80^{o}C until subsequent analyses. DNA extraction followed the protocol outlined previously (Yu & Morrison, 2004). Microbial DNA was analyzed as outlined below.

PCR-DGGE

PCR amplification of the 16S rRNA V3 region was conducted with universal primers U2 and U3 and a 40-60% DGGE analysis were conducted as described by Merrifield *et al.* (2010) (Daniel Lee Merrifield et al., 2010). Dendrograms were created from the DGGE results using ImageJ software.

16S rRNA gene sequencing analysis

Metagenomic analysis of the 16S rRNA V3 and V4 regions were conducted using the Illumina MiSeq system. The following primers were used prior to sequencing (in standard IUPAC nucleotide nomenclature): 16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC The quality of the reads was determined using FastQC and trimmed with Prinseq. QIIME was used for metagenomics quality control and taxon classification and quantification. DESeq2 was used for the differential abundance analysis of the operational taxonomic units (OTUs) identified. Krona charts (Ondov, Bergman, & Phillippy, 2011) were generated from the sequences using the MG-RAST server (Glass & Meyer, 2011) to illustrate the composition of intestinal microbiomes of tilapia fed different inclusion levels of moringa.

Statistical Analysis

The data were analyzed using DESeq2 analysis of deviance (ANODEV) and pair-wise comparisons between treatments. Significance was considered at $P < 0.05$. For diversity

measures, Shannon Diversity Index was calculated for alpha diversity, and Bray-Curtis was used to compare beta diversity along with a principal coordinates analysis.

Results

The analyzed nutrient composition (as fed basis) of *Moringa stenopetala* leaves was found to be as follows: DM 82.81%, Ash 8.60%, Crude Protein 24.50%, Crude Fat 5.36%, Crude Fiber 7.36% and Vitamin C 152mg. Survival, growth rate, feed conversion ratio, protein efficiency ratio of tilapia were not significantly different between treatments (data not presented). The mean percent survival was 94-98% per treatment.

Intestinal Microbiota from PCR-DGGE Results

Intestinal microbiota samples were different between samples, as shown in Figure 2.1, with the highest diversity in the Control and 9% moringa treatments (Table 2.3). However, based on the clustering patterns in the dendrogram (Figure 2), there were no obvious differences between treatments. 16S rRNA gene sequencing analysis was conducted to further elucidate any differences in the intestinal microbiome.

Intestinal Microbiota from Metagenomic Results

Table 2.2 provides a summary of the various bacterial families identified in the 16S rRNA gene sequencing analysis. Of the different families present in the results, 10 have never been reported in tilapia intestinal contents before, to the best of the author's knowledge. These families include: Bryobacteraceae (2017), Corynebacteriaceae (1986 – dyptheria and human saliva), Microbacteriaceae (soil), Propionibacteriaceae (very little known intestinal microbe), Cytophagaceae (found in environmental samples previously), Weeksellaceae (insect intestine and very little else known), Sphingobacteriaceae (Soils and composts – decomposers), Xanthobacteraceae (2005 chemoheterotrophs), Alteromonadaceae (marine), and Chromatiaceae (purple sulfur bacteria).

Intestinal microbiota varied among treatments, as shown in Figures 2.2, 2.3, and 2.4 and Table 2.2. The initial microbiota samples most closely resembled the control (0% moringa) group in the patterns of bacterial orders present. Overall alpha-diversity was not significantly different among treatments (Table 2.3 and Figure 2.4). However, based on the pairwise comparisons and the ANOVA, several microbial groups were significantly different between the moringa and control groups.

As shown in Figure 2.2, the dominant bacterial phyla present were Proteobacteria, Bacteroides, and Actinoids. From the pairwise comparisons (Table 2.4), it was clear that the family Caulobacteraceae was significantly different ($P < 0.05$) between the control and the 9% moringa treatment, making up 7% of the OTUs in this treatment. The Oxalobacteraceae family was significantly different between the control (0% moringa) and the treatments (6, 9, and 12% moringa). Oxalobacteraceae was not present in the 0% treatment and was highest in the 6% and 12% treatments, representing 7% of the OTUs in these treatments. The family Comamonadaceae was significantly different between the control and 6% treatment, making up 15% of the OTUs in this treatment. The phylum Proteobacteria was significantly different ($P < 0.001$) between treatments, with the highest average number of OTUs in the initial sample (3259) and the lowest average OTUs in the 12% moringa inclusion sample at the end of the study (939).

Discussion

A study by Richter et al. (2003) showed that *M. oleifera* leaves could be incorporated as a fishmeal replacement for tilapia up to 10% of the diet without negative effects on growth. In the current study, *M. stenopetala* leaves were incorporated into the diet of hybrid tilapia up to 12% without adverse effects on growth and survival of the fish.

To the best of the author's knowledge, ten of the families of microbes identified in the intestinal tract of tilapia in this study have never been reported previously. Two of these families have been recently described based on their 16S rDNA sequences: Bryobacteraceae in 2017 and Xanthobacteraceae in 2005 so little research has been done on their role in the intestinal microbiome. Microbacteriaceae, Cytophagaceae, and Sphingobacteriaceae are soil microbes that decompose organic material and may either be a product of remaining food particles in the intestine or new decomposers in the tilapia intestinal tract. Xanthobacteracea are chemoheterotrophic bacteria that have not been previously reported in the intestinal tract of animals. Chromatiaceae are purple sulfur bacteria, and Alteromonadaceae are marine microbes that require sodium as a large component of their metabolism. Finally, Corynebacteriaceae, Propionibacteriaceae, and Weeksellaceae are all intestinal microbes that are found in humans, other vertebrates, and insects, respectively. The role of these microbial families in the tilapia intestinal microbiome is unknown at this time.

Based on the pairwise comparisons and the ANOVA of the metagenomic results, several microbial groups were significantly different between the moringa and control groups. Therefore, *Moringa stenopetala* does alter the intestinal microbiota of tilapia.

Previous research stated that Firmicutes were the vast majority of bacteria present in the tilapia samples (Rodiles et al., 2015); however, as shown in Figure 2, the dominant bacterial phyla present were Proteobacteria, Bacteroides, and Actinoids, which is similar to a recent work done in a biofloc system (Kathia, Cienfuegos Martinez, del Carmen, Monroy Dosta Maria, Aida, Hamdan Partida, Jorge, Castro Mejia, Feliz, Aguirre Garrido Jose, Amadeo, 2018). This may be due to the environment and host intestinal ecology of the stock fish raised in Hawaii and kept in a recirculating aquaculture system (Richards et al., 2005; Wong & Rawls, 2012).

The results show a number of significant differences between the control (0% moringa) and the treatment diets (6, 9, and 12% moringa). Oxalobacteraceae was not present in the 0% treatment and was highest in the 6% and 12% treatments, representing 7% of the OTUs in these treatments. *Oxalobacter formigenes* is known to reduce the incidence of kidney stones in humans (Dretler et al., 2008). More work is required to determine the effect of increased Oxalobacteraceae numbers in diets that contain moringa on human and other vertebrate intestinal microbiomes.

The family Comamonadaceae was significantly different between the control and 6% treatment, making up 15% of the OTUs in this treatment. Comamonadaceae abundance is correlated with the ileum IL17 and RORyt mRNA concentration in mice fed high fat diets which led to improved glucose tolerance and fat/lean ratio (Garidou et al., 2015), again suggesting that more research is needed into the effect of *Moringa stenopetala* on intestinal microbes and their ability to affect metabolic diseases such as diabetes and growth and health of fish.

The phylum Proteobacteria was significantly different ($P < 0.001$) between treatments, with the highest average number of OTUs in the initial sample (3259) and the lowest average OTUs in the 12% moringa inclusion sample at the end of the study (939). Proteobacteria have been linked to intestinal inflammation and dysbiosis in humans (Mukhopadhya, Hansen, El-Omar, & Hold, 2012; Shin, Whon, & Bae, 2015); therefore, the inclusion of moringa into tilapia diets may also reduce the proteobacterial load and reduce inflammation, lowering overall costs of tilapia production by reducing health-related costs associated with their production. The closely related

769 species *Moringa oleifera* has already been shown to be an anti-inflammatory agent in humans
770 (Gopalakrishnan et al., 2016; Kooltheat et al., 2014), so it is likely that *M. stenopetala* may have
771 a similar effect. However, more work should be done to investigate the potential anti-
772 inflammatory effects of *M. stenopetala*.
773
774 In conclusion, *Moringa stenopetala* inclusion into the diets of tilapia significantly altered the
775 intestinal microbiome, suggesting that it may be used as a prebiotic in aquaculture. The
776 reduction in proteobacterial levels in the intestine of tilapia suggests that *M. stenopetala* may
777 have anti-inflammatory properties similar to *M. oleifera*. Future studies should investigate the
778 anti-inflammatory properties of *M. stenopetala* by investigating the histological effects of
779 moringa in the diets of tilapia.
780

Table 2.1 Ingredient composition and nutrient content of diets fed in the study

Item	Diets			
	Control	6% Moringa	9% Moringa	12% Moringa
Ingredient, g/kg				
Soybean meal (45% CP)	100.0	100.0	100.0	100.0
Moringa leaf	-	60.0	90.0	120.0
Corn	185.4	180.9	143.9	116.9
Fish and meat meal	200.0	200.0	200.0	200.0
Profine®[1]	250.0	230.0	220.0	210.0
Cassava flour	180.0	150.0	190.0	200.0
Soybean oil	10.0	10.0	-	-
Molasses	20.0	20.0	10.0	10.0
Lysine	12.0	9.0	7.0	5.0
Methionine	4.0	3.5	3.0	3.0
Threonine	3.5	1.5	1.0	-
Choline chloride	15.0	15.0	15.0	15.0
Phytase	0.10	0.10	0.10	0.10
Vitamin mix	10.0	10.0	10.0	10.0
Mineral mix	10.0	10.0	10.0	10.0
Analyzed composition (as fed, g kg^{-1}) (n=3)				
Dry matter	89.03	89.12	88.73	88.58
Ash	6.43	7.18	7.19	7.44
Crude protein	33.57	34.15	33.64	33.5
Crude fat	5.74	6.56	5.28	5.16
Crude fiber	6.43	4.53	5.01	4.89
NDF	11.40	13.53	11.00	13.52
ADF	4.77	5.81	5.92	7.01
Vitamin C	96.4	105.3	136.8	163.2
Gross energy (kcal/kg)	3205	3217	3226	3267

1. Profine® Powdered Soy Protein Concentrate was purchased from the DuPont® Feed company, Wilmington, DE, USA

Ingredients are expressed as g per kg^{-1} diet. Dietary codes: 6% = 6% moringa inclusion; 9% = 9% moringa inclusion; 12% = 12% moringa inclusion.

Table 2.2 List of bacterial genera and their corresponding sample presence. Samples are taken from the intestine of hybrid tilapia fed either 0, 6, 9, or 12% ground moringa leaf inclusion in the diet or from the initial tilapia samples prior to experimental feeding.

Phylum	Class	Order	Family	Genus	Samples
Acidobacteria	Soilbacteres	Soilbacterales	Bryobacteraceae	Unknown	6, 9, 12%
Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	0, 9%, Initial
			Microbacteriaceae	Unknown	6, 9, 12%
				Clavibacter	0, 6, 9, 12%
			Propionibacteriaceae	Pripionibacterium	0%, Initial
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Initial
	Cytophagia	Cytophagales	Cytophagaceae	Unknown	6, 9, 12%
				Emticicia	6%
				Runella	0, 6, 9, 12%
	Flavobacterila	Flavobacteriales	Flavobacteriaceae	Flavobacterium	All
			Weeksellaceae	Cloacibacterium	6, 9%
	Sphingobacteriia	Sphingobacteriales	Unknown	Unknown	0, 6, 9, 12%
			Sphingobacteriaceae	Unknown	0, 6, 12%
			Sphingobacteriaceae	Sphingobacterium	Initial
	Saprospirae	Saprospirales	Chitinophagaceae	Sediminibacterium	0, 6, 9, 12%
			Saprospiraceae	Unknown	0, 6, 9%
				Haliscomenobacter	0, 9, 12%
Chlorobi	OPB56	Unknown	Unknown	Unknown	6, 9, 12%
Cyanobacteria	Unknown	Unknown	Unknown	Unknown	0, 6%

Firmicutes	Clostridia	Clostridiales	Unknown	Unknown	0, 6, 9% Initial
Fusobacteria	Fusobacterila	Fusobacteriales	Fusobacteriaceae	Cetobacterium	Initial
Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae	Unknown	0, 9, 12%
		Pirellulales	Pirellulaceae	Unknown	0, 12%
Proteobacteria	Alphaproteobacteria	Unknown	Unknown	Unknown	0, 6, 9%
		Caulobacterales	Caulobacteraceae	Unknown	Initial
				Asticcacaulis	6, 9%
				Caulobacter	6, 9, 12%
				Mycoplana	Initial
		Rhizobiales	Unknown	Unknown	0, 6, 12%, Initial
			Rhizobiaceae	Shinella	Initial
			Xanthobacteraceae	Ancylobacter	6, 9, 12%
		Rhodobacterales	Rhodobacteraceae	Rhodobacter	0, 6, 9%, Initial
		Rhodospirillales	Rhodospirillaceae	Unknown	All
		Sphingomonadales	Sphingomonadaceae	Novosphingobium	All
				Sphingomonas	Initial
				Sphingopyxis	Initial
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	0, 9%
			Comamonadaceae	Unknown	6, 9, 12%
			Oxalobacteraceae	Polynucleobacter	6, 9, 12%
	Gammaproteobacteria	Alteromondales	Alteromonadaceae	Cellvibrio	0, 6, 9, 12%

			Chromatiaceae	Rheinheimera	6, 9%
		Enterobacteriales	Enterobacteriaceae	Escherichia	Initial
		Legionellales	Unknown	Unknown	Initial
		Pseudomonadales	Moraxellaceae	Acinetobacter	Initial
			Pseudomonadaceae	Pseudomonas	Initial
		Xanthomonadales	Xanthomonadaceae	Pseudoxanthamonas	12%, Initial
Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Unknown	0, 9, 12%
	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Unknown	9, 12%

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Table 2.3 Shannon Diversity Index results for the 16S rRNA gene sequencing analysis of the intestinal microbiota of tilapia fed diets containing different inclusion levels of moringa (0%, 6%, 9%, 12%). Initial microbiota analysis was included for comparison.

Treatment	Bacterial Orders	Reads	H’
Initial	21	6260	2.62
0%	27	5603	2.97
6%	20	7397	2.49
9%	23	9125	2.94
12%	21	8712	2.37

Table 2.4 Pairwise comparisons of microbial diversity between the control group (0% moringa inclusion) and the treatments (6%, 9%, 12% moringa inclusion). Only significant differences are reported here. For a full list of microbial orders present in the samples, see Table 5 and Figure 4.

Treatment	Family	0% OTU	Treatment OTU	Significance
6% Moringa	Comamonadaceae	0	688	0.03
	Oxalobacteraceae	0	223	<0.001
9% Moringa	Caulobacteraceae	0	364	0.001
	Oxalobacteraceae	0	104	0.056*
12% Moringa	Oxalobacteraceae	0	323	<0.001

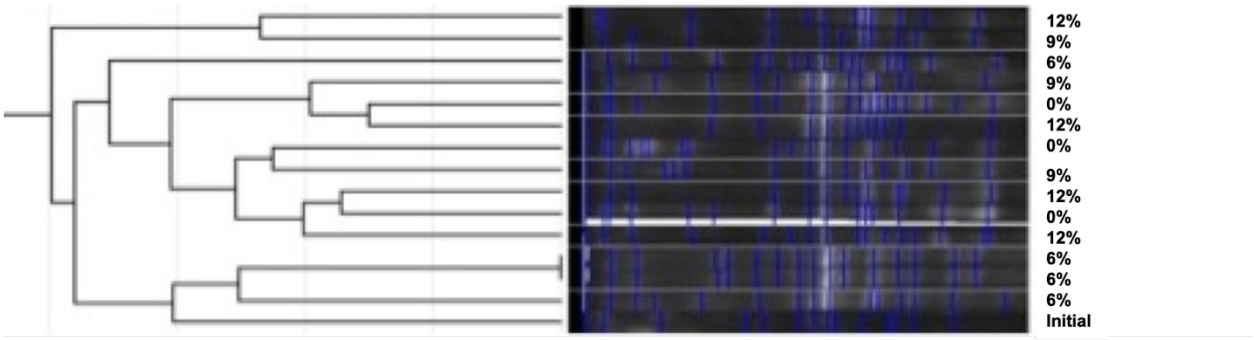

Figure 2.1 PCR-DGGE Dendrogram results of intestinal microbiota from diets with different inclusion levels of moringa: Initial (Initial microbiota samples before feeding); 0% (Control with no moringa inclusion): 6% moringa inclusion; 9% moringa inclusion; and 12% moringa inclusion.

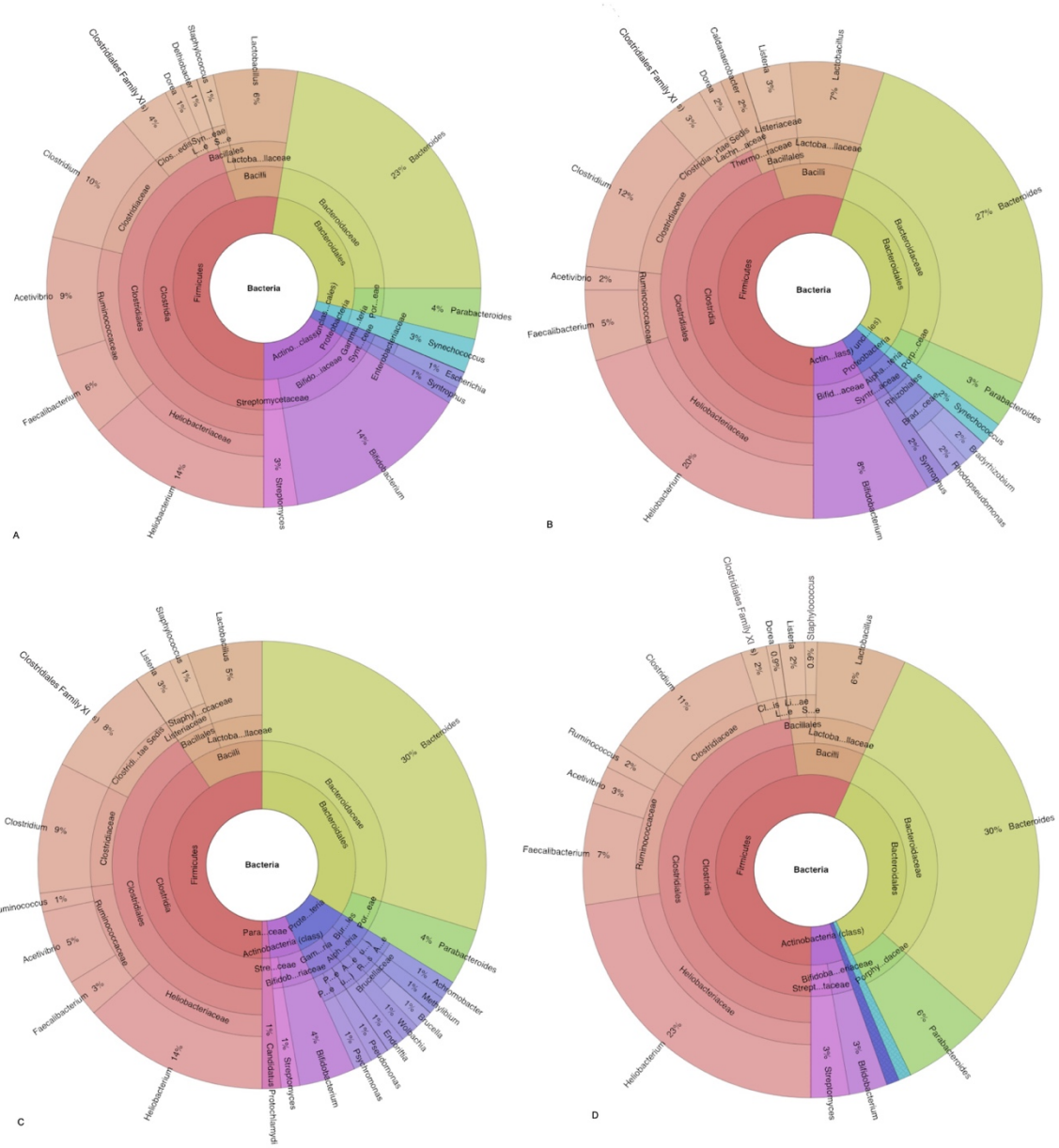

Figure 2.2 Krona Charts of Bacterial Family Diversity in the microbiota of hybrid tilapia fed different treatment diets: Control (0% Moringa, A), 6% Moringa (B), 9% Moringa (C), 12% Moringa (D).

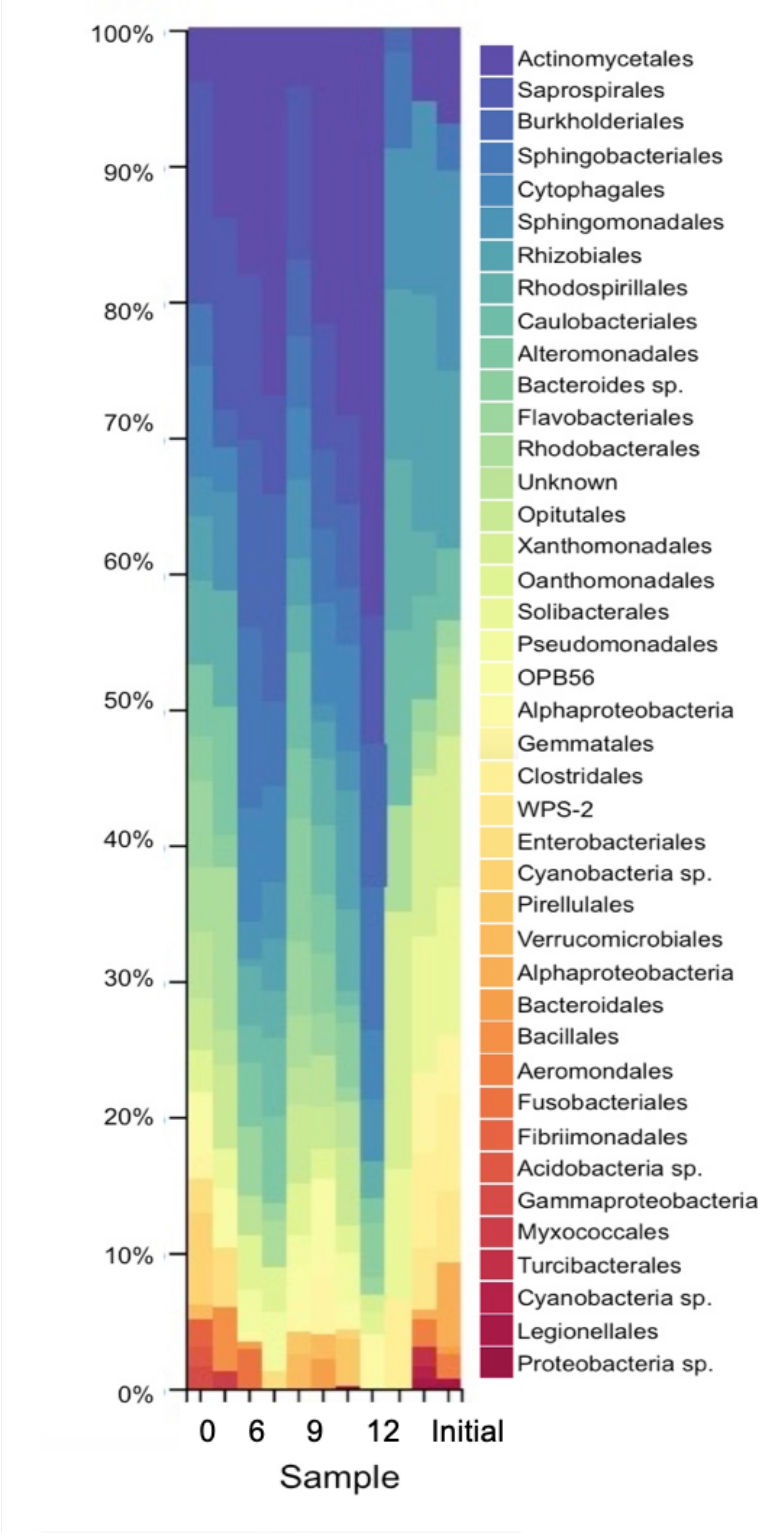

Figure 2.3 Relative frequency of bacterial orders per treatment highlighting the different microbiota present with varying moringa inclusion levels (0, 6, 9, and 12%) in hybrid tilapia diets compared to the initial microbiota of hybrid tilapia before first feeding of trial diets.

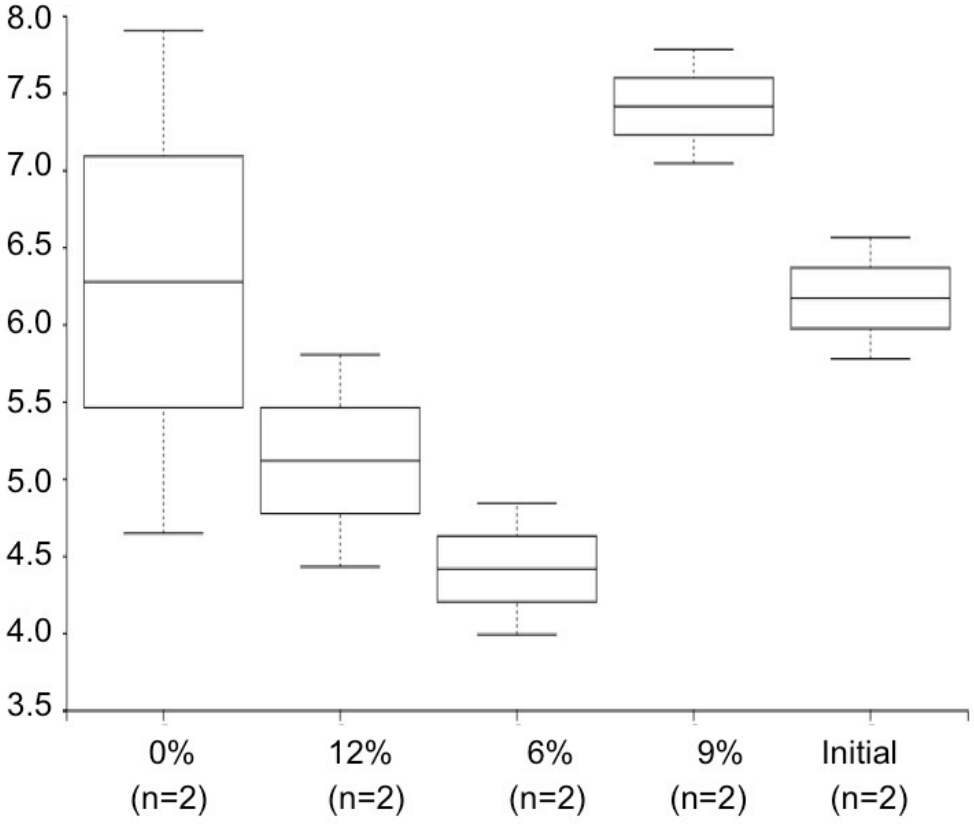

Figure 2.4 Alpha Diversity of treatments using Faith's Phylogenetic Diversity. Kruskal Wallis results showed no significant differences between treatment alpha diversity.

CHAPTER 3 CASSAVA CHIPS AS A PREBIOTIC IN TILAPIA AQUACULTURE

Abstract

In order to increase aquaculture production while reducing production costs, alternative ingredients must be investigated to reduce the dependency on conventional energy sources in aquaculture feeds. Like prebiotics, feed ingredients with high fiber and resistant starch also modulate the intestinal microbiota of the host while providing nutritional value for growth performance. Tilapia (*Oreochromis sp.*) are the most widespread aquaculture species in the world due to their relative fecundity, omnivorous feeding habits, and tolerance of marginal growing conditions, making them ideal study species for alternative feed ingredients. This study investigated the use of sun-dried cassava (*Manihot esculenta*) chips as corn replacement to reduce feed cost while maintaining production levels of hybrid tilapia (*O. niloticus x O. mossambicus*). Four hundred tilapia fingerlings (~10g initial body weight) were randomly and equally placed in 20 tanks and fed with one of the 5 diets with 0, 4.38, 8.75, 17.5, and 26.25% cassava inclusion in basal diets for 12 weeks. Feed intake and body weight was recorded weekly and intestinal samples were collected monthly and processed for microbiota analysis using PCR-DGGE and 16S rRNA gene sequencing analysis. Tilapia growth performance was not significantly different among treatments (P>0.05), suggesting that cassava can be incorporated up to 26.25% into the tilapia diets without negative impact. Additionally, cassava inclusion into the tilapia diets significantly altered the intestinal microbiota, which can be used as a strategy to modulate gut health of Tilapia.

Keywords: Cassava, Microbiota, Intestinal Microbiome

Introduction

Aquaculture feed is one of the primary costs in production and a number of studies have been conducted to determine the optimal feed for growth and development of aquatic animals, such as tilapia, at minimal cost. Tilapia (*Oreochromis* sp.) is the second most farmed fish group worldwide and over the past decade has quadrupled in production, largely due to their many characteristics conducive to aquaculture conditions as well as to the high marketability and relatively stable market prices (Ng & Romano, 2013). Production of tilapia in 2015 exceeded 5.7 million tons worldwide with a value of over $9 billion USD and has continued to subsequently increase (FAO, 2016). The ability to maximize production of tilapia at decreased cost will continue this upward trend while providing protein for an increasing human population.

Corn is the major feed ingredient, providing energy in the diets of tilapias. Its diverse uses in food, feed, and biofuel production has led to variability in cost and availability. Thus, it is imperative to explore and evaluate alternative feed ingredients to replace corn in the fish diets for sustainable and cost-effective fish production. Cassava (*Manihot esculenta*) is one alternative to corn, as it is rich in starch content. It is available globally with 70% of the production coming mostly from Tropical countries like from Nigeria, Brazil, Thailand, Indonesia, and the Democratic Republic of the Congo. Production levels in 2017 were projected at 278 million tons worldwide, with the majority of international flow confined to East and Southeast Asia (FAO, 2015). Cassava root chips are rich in carbohydrate content, and thus may be used as a prebiotic in aquaculture. However, it is a poor source of protein and contains anti-nutritional factors like cyanogenic glucosides, linamarin, and lotaustralin, which on hydrolysis yield hydrocyanic acid (HCN). HCN toxicity might be limiting factor of using cassava in fish diets (Oke, 1978). The HCN in cassava can be considerably reduced to the acceptable limit by boiling, drying, grating, soaking, fermentation, or combination of these processes (Chhay, Borin, Sopharith, Preston, & Aye, 2010; Ravindran, 1993). Cassava, having a high moisture content, is usually dried in the sun. Sun drying is also a more cost-effective and energy efficient method as compared to oven-drying. Moreover, sun drying is more effective than oven-drying at reducing the HCN level of cassava chips (Mestres & Rouau, 1997), which is a major concern of using cassava in animal diets. There is also variation in the nutrient profile of cassava chips due to several factors including type of cassava and agro-climatic condition where it was grown (Coursey & Halliday, 2017; Mestres & Rouau, 1997; Oke, 1978; Ravindran, 1993).

Animal scientists advocate for the use of cassava root and byproducts in animal feeding programs due to the ease of cultivation, particularly in dry climates (Coursey & Halliday, 2017; Lukuyu, Okike, Duncan, Beveridge, & Blümmel, 2014). Previous studies utilizing cassava in tilapia feed have found inclusion levels of around 10% allow for the maintenance of production levels without deleterious health effects on the tilapia (Chhay et al., 2010; Sena et al., 2012; Tram, Ngoan, Hung, & Lindberg, 2011). Some of the researches have also shown positive results with inclusion up to 50% (Lukuyu et al., 2014). Cassava is rich in carbohydrates, particularly starches, and can provide energy and protein for tilapia. It is also currently used as a binder in many feeds (Coursey & Halliday, 2017).

The intestinal microbiota of fish are directly impacted by the feed ingredients used in the fish diet, particularly the carbohydrate sources (Haygood & Jha, 2018; Rurangwa et al., 2009; Tran-Duy, Smit, van Dam, & Schrama, 2008). Nondigestible feed ingredients are known as prebiotics and can alter the intestinal microbiome of the host that consumes them. This can provide host benefits including: modulation of blood lipid levels, gastrointestinal and systemic immunomodulation, provide energy for intestinal cell proliferation, and improve intestinal barrier function, among other benefits (Dimitroglou et al., 2011; Haygood & Jha, 2018; Llewellyn et al., 2014; Sukanta K Nayak, 2010; Roberfroid, 2007). Cassava is known for its high starch content and provides a number of prebiotic ingredients for tilapia microbiota (Chhay et al., 2010; Coursey & Halliday, 2017; Pedrotti et al., 2015b; Sena et al., 2012; Tram et al., 2011)

The effect of cassava on the intestinal microbiome of tilapia has been studied using culture-based and PCR-DGGE techniques, but to the authors' knowledge, there have not been any studies investigating the effect of cassava on the microbiome using next-generation sequencing methods. The purpose of this study was to evaluate the effect of dietary inclusion of *Manihot esculenta* sun-dried chips on the intestinal microbiome in hybrid tilapia. We hypothesized that the incorporation of cassava chips in tilapia diets will significantly alter the intestinal microbial composition.

Materials and methods

All animal procedures were conducted in accordance with the approval (protocol #13-1639) from the Institutional Animal Care and Use Committee (IACUC) of the University of Hawaii, Honolulu, HI, USA.

Preparation of Cassava chips

Locally produced and processed (sun dried) cassava root chips with peel were used in this study. The cassava chips were ground through sieve size of 3/16. Prior to diet formulation, proximate and other nutrient composition of cassava chips were determined. The nutrient profile was used from a previous study conducted in our lab.

Feeding trial

A total of 400 tilapia (~20-30g) were randomly and equally allocated into the 20 freshwater tanks (20 fish per tank) located at Magoon research facility of UH Manoa. Each tank was labeled randomly as one of the five dietary experimental groups. During the acclimation period of 7

days, all tilapia were offered the control diet twice a day (8 am and 4 pm) up to 5% of their body weight per day. During the experiment period, tilapia were fed the formulated diets by hand 2 times a day (8 am and 4 pm) up to 5% of their body weight per day every day for 84 days. Water quality parameters (nitrates, nitrites, ammonia, pH, temperature, dissolved oxygen) were measured weekly before feeding and maintained to have healthy water quality.

Water Quality

Water quality parameters (pH, nitrates, nitrites, ammonia, temperature, and dissolved oxygen, DO) were monitored weekly to ensure they were within the optimal range for tilapia. The DO concentration, temperature, and pH were measured routinely using the HQ40d Portable Water Quality Lab Package (Hach, Loveland, CO, USA). Total ammonia nitrogen (TAN, NH_3-N) was measured using the reaction kit Ammonia TNTplus, ULR (TNT 830, Hach, Loveland, CO, USA). Nitrite-N (NO_3-N) was measured using the reaction kit Nitrite TNTplus, LR (TNT 839, Hach, Loveland, CO, USA). Nitrate-N (NO_2-N) was measured using the reaction kit Nitrate TNTplus, HR (TNT 836, Hach, Loveland, CO, USA). Water temperature was maintained at 22.5±0.8^{o}C, DO was maintained at 10.7±0.7 mg/L, pH was 7.9±0.25, NH_3-N was 3.4±3.6 ppm, NO_3-N was 2.97±2.0 ppm, and NO_2-N was 12.4±22.7 ppm. A 12 h light／12 h dark photoperiod was maintained throughout the animal study period.

Experimental diet

The ground cassava chips sample was subjected to proximate analysis according to the Association of Official Analytical Chemists standard procedures (AOAC, 2007) with specific methods as follows: DM (135°C for 2 h, AOAC 930.15); Crude protein (CP) analysis (AOAC 976.05) by determining nitrogen (N) using LECO TruSpec CN analyzer (LECO Corp., St. Joseph, MI, USA) and N multiplied by 6.25 to get the % CP; crude fiber (AOAC 978.10), crude fat (AOAC 920.39; using Soxhlet apparatus and petroleum ether), and ash (AOAC 942.05). Five isocaloric and isonitrogenous corn-soybean meal based diets were formulated (Table 3.1) to meet or exceed the nutritional requirements of tilapia fry (NRC, 1993). The diets varied in cassava chips in the amounts of 0% (control, T1), 4.38% (T2), 8.75% (T3), 17.50% (T4), and 26.25% (T5) replacing 0, 12.5, 25, 50, and 75% of corn, respectively and were balanced with other ingredients. These treatments were allotted to tilapia in a completely randomized design. All diets were analyzed following the same methods as the cassava chip samples (described above); the ingredient composition and analyzed nutritional values of all diets are presented in Table 3.1.

968
969 *Intestinal microbiota sampling*
970 Tilapia intestinal microbiota was sampled monthly and the intestinal samples from two fish per
971 tank were pooled (four samples per treatment). The fishes were euthanized with 0.6 g l^{-1}
972 Tricaine Methanesulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA), buffered
973 with 0.12 g l^{-1} sodium bicarbonate in water originating from the corresponding rearing tank.
974 Subsequently, fish were rinsed with 70% ethanol and sterile water before dissecting out
975 aseptically the gut under a dissection microscope. Whole gut samples were flash frozen in liquid
976 nitrogen and stored individually at 80^{o}C until subsequent analyses. DNA extraction followed the
977 protocol outlined by Yu and Morrison (2004) (Yu & Morrison, 2004). Microbial DNA was
978 analyzed as outlined below.
979
980 *PCR-DGGE*
981 PCR amplification of the 16S rRNA V3 region was conducted with universal primers U2 and U3
982 and a 40-60% DGGE analysis were conducted as described (Merrifield et al., 2010).
983 Dendrograms were created from the DGGE results using ImageJ software.
984
985 *16S rRNA gene sequencing analysis*
986 Metagenomic analysis of the 16S rRNA V3 and V4 regions were conducted using the Illumina
987 MiSeq system. The following primers were used prior to sequencing (in standard IUPAC
988 nucleotide nomenclature): 16S Amplicon PCR Forward Primer =
989 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
990 16S Amplicon PCR Reverse Primer =
991 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
992 The quality of the reads was determined using FastQC and trimmed with Prinseq. QIIME was
993 used for metagenomics quality control and taxon classification and quantification. DESeq2 was
994 used for the differential abundance analysis of the operational taxonomic units (OTUs)
995 identified. Krona charts (Ondov et al., 2011) were generated from the sequences using the MG-
996 RAST server (Glass & Meyer, 2011) to illustrate the composition of intestinal microbiomes of
997 tilapia fed different inclusion levels of moringa.
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Statistical Analysis

Data were analyzed by ANOVA using Mixed procedure of SAS (SAS 9.2, SAS Institute Inc., Cary, NC). Significant differences among treatments were assessed by Tukey's test. A significant level of P less than equal to 0.05 was used to declare difference.

The metagenomic data were analyzed using DESeq2 analysis of deviance (ANODEV) and pair-wise comparisons between treatments. Significance was considered at $P < 0.05$. For diversity measures, Shannon Diversity Index was calculated for alpha diversity, and Bray-Curtis was used to compare beta diversity along with a principal coordinates analysis.

Results

The analyzed nutrient composition (as fed basis) of *M. esculenta* chips was as follows: Dry Matter 90.06%, Ash , Crude Protein , Crude Fat , and Crude Fiber . Survival and growth parameters of tilapia were not significantly different between treatments (data not presented). Survival, growth rate, average daily feed intake, feed conversion ratio, protein efficiency ratio of tilapia were not significantly different between treatments (data not presented). The mean percent survival was 94-99% per treatment.

Intestinal Microbiota from PCR-DGGE Results

Intestinal microbiota samples were different between groups, as shown in Figure 3.1. Clustering in the dendrogram shows similarities between the control group and the 12.5% and 25% cassava feed, while the 50% and 75% cassava feed clustered together. To better elucidate these differences, a 16S rRNA gene sequencing analysis was performed on the samples.

Intestinal Microbiota from Metagenomic Results

Table 3.2 provides a summary of the species counts and Shannon Diversity Index. The Control group had the lowest index of diversity (1.315), while the 50% cassava group had the highest (2.329). The highest species count was in the 75% cassava sample with 596 species identified, while the Control had the lowest at 436.

In Figure 3.2, the top 30 bacterial families are summarized per sample and the full Krona Charts are shown in Figure 3.3. The four main Bacterial Phyla represented in all treatments included Proteobacteria, Firmicutes, Planctomycetes, and Actinobacteria. The most common bacterial

families were the Gemmataceae and Streptococcaceae for all samples but the 50% cassava sample which had Fusobacteriacea as the dominant family.

The Principle Coordinate Analysis of the metagenomic results by Bacterial Families indicates clustering of 75% and 12.5% Cassava and the 25% Cassava and Control (Figure 3.4).

Discussion

Based on the results of this study, cassava chips can be incorporated to replace up to 75% of the corn meal in tilapia diets without negative effects on growth or health of the fish. It has been well documented that feed alters the intestinal microbiome within fishes, particularly by altering the carbohydrate content of the diet (Haygood & Jha, 2018; D. Merrifield & Ringø, 2014; Sukanta K Nayak, 2010; Ringø et al., 2010). According to the Shannon Diversity Index results, with increasing cassava chip inclusion in the diet there was an increase in diversity up to 50% corn replacement (17.5% total inclusion); therefore, cassava chip inclusion in the diet as a corn replacement alters the intestinal microbiota of tilapia.

The four main bacterial Phyla represented in all treatments included Proteobacteria, Firmicutes, Planctomycetes, and Actinobacteria, which supports previous studies regarding the dominant phyla in fish microbiomes based on metagenomic analyses (Tarnecki, Burgos, Ray, & Arias, 2017a). Within these phyla, the dominant bacterial families included Gemmataceae and Streptococcaceae for all samples but the 50% cassava sample which had Fusobacteriacea as the dominant family. To the authors' knowledge, the only report of Gemmataceae in tilapia is from Gaikwad, Shouche, and Gade (2017). There is very little known about the effects of this bacterial family in the intestinal microbiome and more research should be conducted into its potential importance.

The Streptococcaceae family was significantly different between treatments ($P<0.01$) with the highest count (22484 OTUs) in the control and lowest counts in the 50% Cassava (9272 OTUs) and 75% Cassava (10458 OTUs) treatments, suggesting a decrease of Streptococcus species with increased cassava chip inclusion in the diet. *Streptococcus sp*. are known pathogens of freshwater species, including tilapia with *Streptococcus agalactiae* being one of the most well studied (Amal & Zamri-Saad, 2011; Ng, Koh, Sudesh, & Siti-Zahrah, 2009).

Of the other bacterial families detected, Microbacteriaceae and Rhizobiaceae are soil microbes that decompose organic material and may either be a product of remaining food particles in the intestine or new decomposers in the tilapia intestinal tract and were found in higher abundance in the cassava diets compared to the control. Xanthobacteracea are chemoheterotrophic bacteria that have not been previously reported in the intestinal tract of animals and decreased in abundance with increasing cassava inclusion compared to the control. Finally, Methylobacteriaceae were significantly different between the 75% Cassava treatment (6049 OTUs) and the Control (2566 OTUs) ($P<0.01$). This family has been described as a beneficial species in the tilapia intestinal microbiome in previous studies, suggesting that the increase of cassava in the diet may beneficially effect tilapia (Zheng et al., 2018).

In conclusion, *Manihot esculenta* inclusion into the diets of tilapia did not negatively impact the growth, but significantly altered the intestinal microbiome, suggesting that it may be used in aquaculture. The increase of the Methylobacteriaceae family in the tilapia intestinal microbiota suggest that cassava inclusion in the diet may beneficially affect tilapia. Additionally, the reduction in levels in Streptococcaceae in the intestine of tilapia suggests that *M. esculenta* may reduce pathogenic load in farmed tilapia. Future studies should investigate the histological effects of cassava in the diets of tilapia to better elucidate its potential as a prebiotic.

Table 3.1 Ingredient composition and nutrient content of diets fed in the cassava study

Ingredients	**CSV0**	**CSV12.5**	**CSV25**	**CSV50**	**CSV75**
Corn	35	30.63	26.25	17.5	8.75
SBM	35	35	35	35	35
Cassava chips	0	4.38	8.75	17.5	26.25
Profine[1]	11.54	11.54	13	13.04	14.04
Soybean oil	7.5	7	6.04	4	2
Molasses	5	5	5	6	6
Others	5.96	6.46	5.96	6.96	7.96
Total	100	100	100	100	100
Calculated content, %					
DE, Kcal/kg	3450	3485	3514	3525	3550
CP	26.94	26.73	27.45	27.13	27.35
Ether extract	9.13	8.64	7.74	5.79	3.88
Crude fiber	4.68	3.2	3.23	3.65	4.04
ADF	4.47	3.85	3.95	4.2	4.59
NDF	9.97	6.42	6.69	6.44	7.64
Lysine	2.06	2.06	2.06	2.05	2.05
Threonine	1.36	1.35	1.37	1.36	1.36
Methionine	1	1	1.02	1.01	1.01
Choline (mg/kg)	1314	1293	1275	1254	1216
Ca	0.153	0.171	0.173	0.217	0.254
P	0.315	0.3125	0.297	0.29	0.284

1. Profine® Powdered Soy Protein Concentrate was purchased from the DuPont® Feed company, Wilmington, DE, USA

Ingredients are expressed as g per kg^{-1} diet. Dietary codes: CSV0 = Control, CSV12.5 = 12.5% corn replacement, CSV25 = 25% corn replacement, CSV 50% = 50% corn replacement, CSV75 = 75% corn replacement.

Table 3.2 Number of reads, species counts, and Shannon Diversity Index values per sample.

Sample	Number of Reads	Species Counts	Shannon Diversity Index
Control	99,931	436	1.315
12.5% Cassava	115,882	511	1.566
25% Cassava	88,329	536	1.361
50% Cassava	102,742	543	2.329
75% Cassava	114,572	596	1.908

Figure 3.1 Results of the PCR-DGGE analysis of cassava samples replacing 0%, 12.5%, 25%. 50%, and 75% of corn in the diet of tilapia.

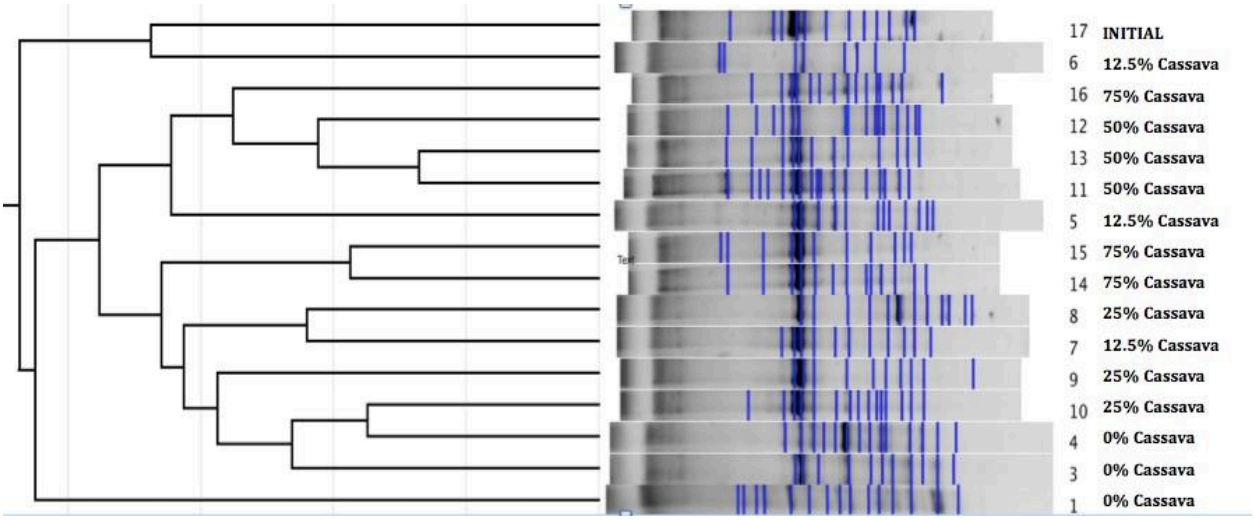


Figure 3.2 Top 30 Family classification results per sample. Samples are labeled as follows: Cass0 = Control; Cass12.5 = 12.5% Cassava; Cass25 = 25% Cassava; Cass50 = 50% Cassava; Cass75 = 75% Cassava.

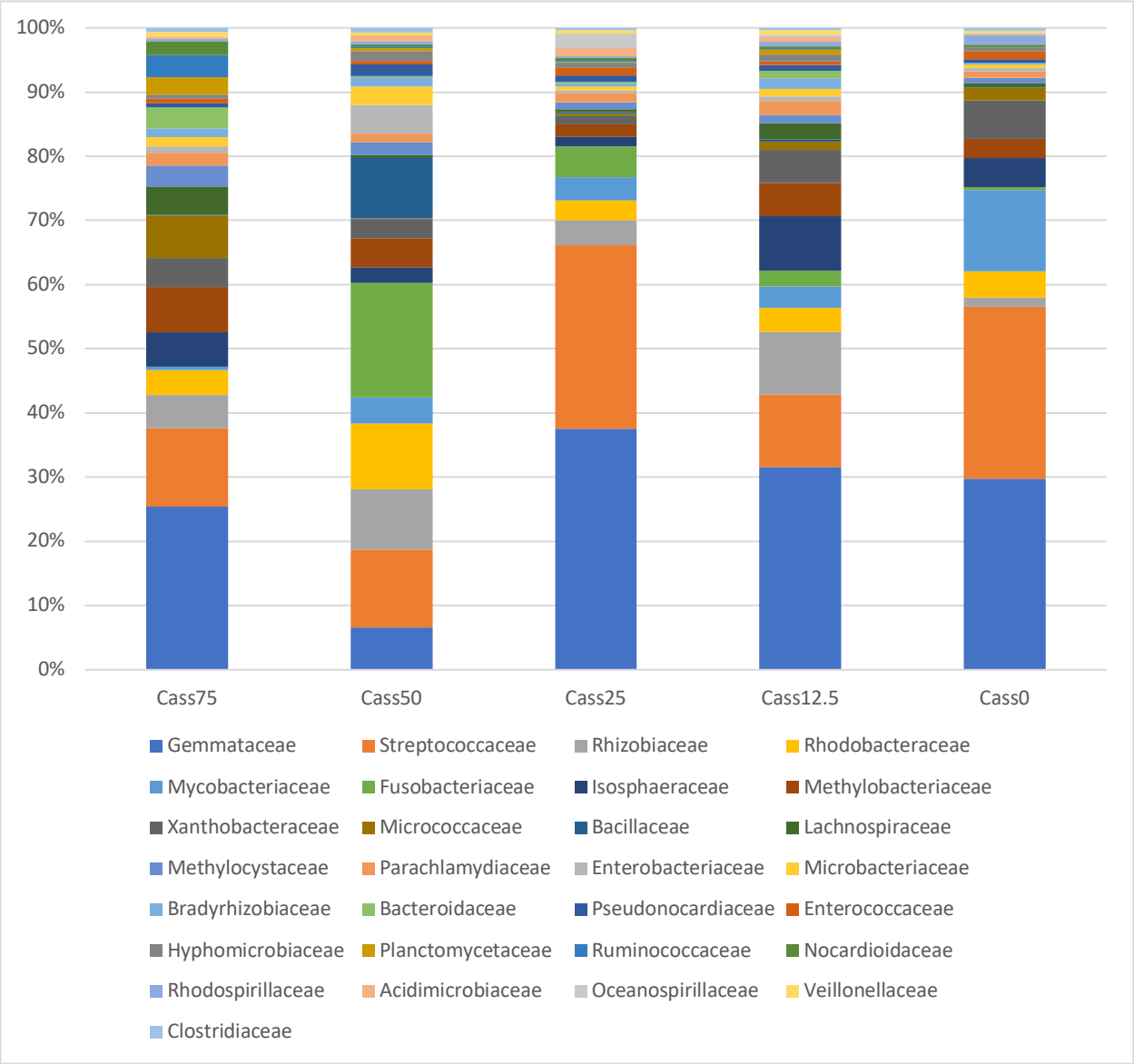
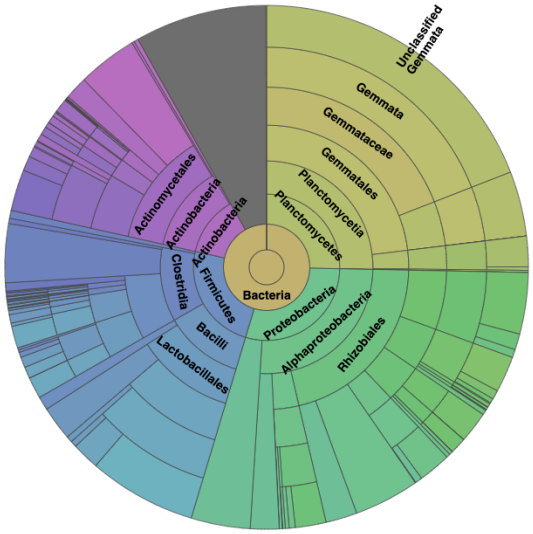
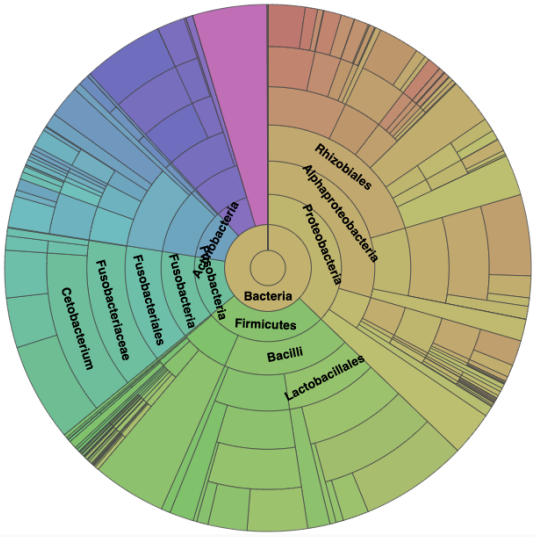


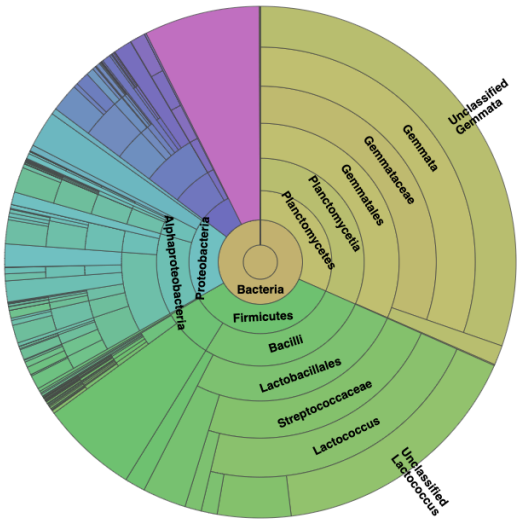
Figure 3.3 Krona charts of bacterial classifications by sample.



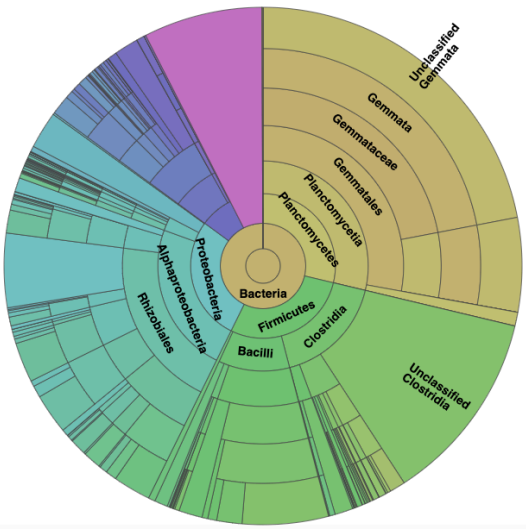
a) 75% Cassava



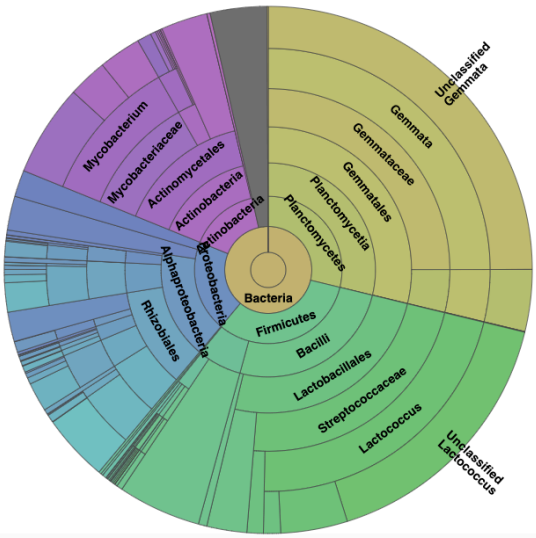
b) 50% Cassava



c) 25% Cassava

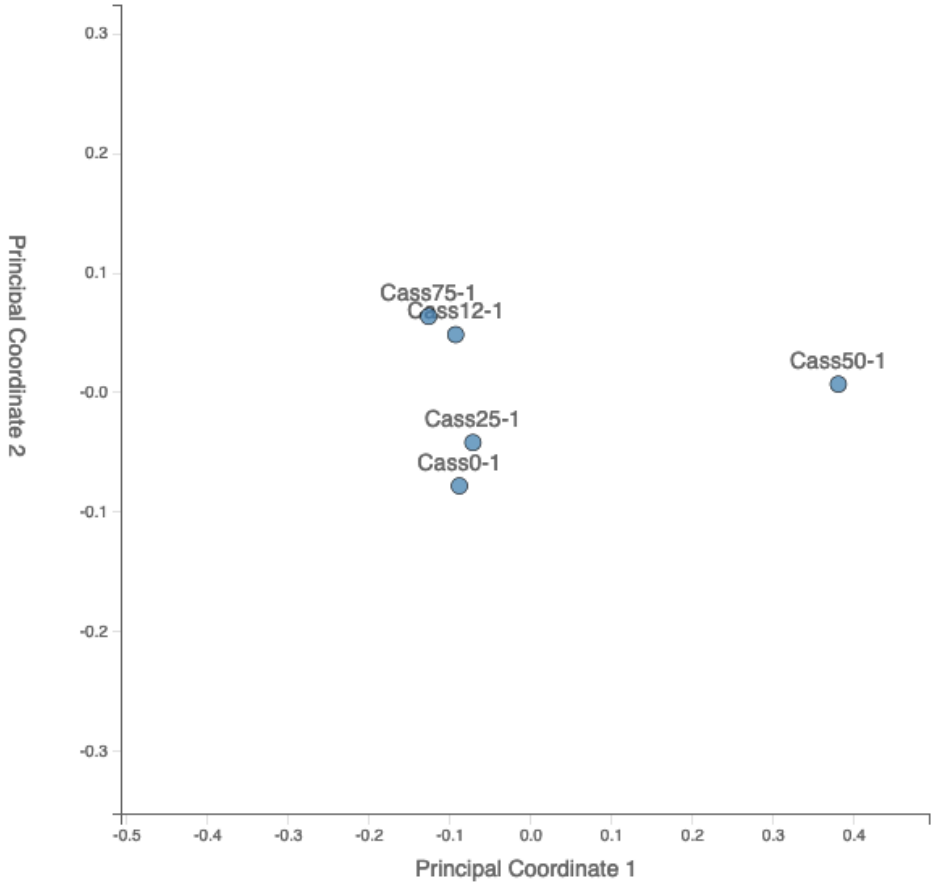


d) 12.5% Cassva


Unclassified Gemmata
Gemmata
Gemmataceae
Gemmatales
Planctomycetia
Planctomycetes
Bacteria
Firmicutes
Bacilli
Lactobacillales
Streptococcaceae
Lactococcus
Unclassified Lactococcus
Rhizobiales
Alphaproteobacteria
Proteobacteria
Actinobacteria
Actinomycetales
Mycobacteriaceae
Mycobacterium

e) Control

Figure 3.4 Principle Coordinate Analysis (PCoA) of the normalized relative abundance of all samples based on family-level classifications. Samples are labeled as follows: Cass0-1 = Control; Cass12-1 = 12.5% Cassava; Cass25-1 = 25% Cassava; Cass50-1 = 50% Cassava; Cass75-1 = 75% Cassava.



CHAPTER 4 THE EFFECT OF MICROALGAE AS A FISHMEAL AND FISH OIL REPLACEMENT ON THE INTESTINAL MICROBIOTA OF TILAPIA

Abstract

The aquaculture industry has explored alternative protein and lipid sources for aquaculture feed to reduce costs and promote sustainability. Microalgae is a desirable feed ingredient because it is grown in high volume and can be selected for high levels of lipids and protein. Additionally, microalgae have been recently explored as a prebiotic to promote the health of fish species through the modulation of the intestinal microbiota. This study investigated the use of microalgae as fishmeal and fish oil replacements and its effect on the intestinal histology and microbiota of hybrid tilapia (*O. niloticus x O. mossambicus*). Feed intake and body weight was recorded weekly and intestinal samples were collected monthly and processed for microbiota analysis using metagenomics and histological analysis of the intestinal epithelia. Tilapia growth performance was significantly higher in the 100% algae-based diet compared to the fishmeal and fish oil control ($P>0.05$), suggesting that microalgae can completely replace fishmeal and fish oil in the tilapia diets without negative impact. Microalgae inclusion into the tilapia diets did not significantly alter the overall intestinal microbiota. However, several species were significantly different between treatments, most notably an increase in *Parabacteroides goldsteinii* in the 100% algal inclusion. *P. goldsteinii* is a known beneficial microbe in the human GI tract, thus the inclusion of algae in the diets may be used as a strategy to modulate the gut health of Tilapia. Additionally, several novel species were reported in tilapia including the two most common species across all treatments, *Mycobacterium pinnipedii*, a closely related species to the tuberculosis-causing *Mycobacterium tuberculosis*, and the non-tuberculosis causing, *Mycobacterium brasiliensis*. Histological results did not show a significant difference between treatments, suggesting that algae inclusion in the diet does not negatively affect the health of tilapia.

Keywords: Microalgae, Microbiota, Intestinal Microbiome, Intestinal Histology

Introduction

The largest costs in aquaculture production include the fish feed and maintenance of fish health through disease prevention and treatment (El-Sayed & Tacon, 1997; FAO, 2016; Hardy, 2010; Haygood & Jha, 2018; S. K. Nayak, 2010; Olsen & Hasan, 2012). Many fish feeds include

protein content from wild caught fish, which has been shown to be unsustainable (El-Sayed & Tacon, 1997; Naylor et al., 2009; New & Wijkström, 2002). Therefore, the aquaculture industry has explored alternative protein and lipid sources for aquaculture feed. Since the European Union 2006 ban on the use of antibiotics as a growth promoter in agriculture and aquaculture, research has been directed into the use of prebiotics and probiotics to modulate the intestinal microbiota of aquaculture species (Gifstad et al., 2010; Haygood & Jha, 2018; S. K. Nayak, 2010; Sukanta K Nayak, 2010). Prebiotics are compounds in food that induce the growth or activity of beneficial microorganisms such as bacteria and fungi in the intestinal tract of the species of interest. To date, the majority of prebiotic research has involved the use of non-digestible oligosaccharides and inulin (Llewellyn, Boutin, Hoseinifar, & Derome, 2014; Merrifield & Ringø, 2014; Ringø et al., 2010).

Modulation of the intestinal microbiota of aquaculture species through the use of prebiotics not only benefits the health of the host, but also reduces costs to aquaculture production (Merrifield & Ringø, 2014). Like prebiotics, feed ingredients with high fiber and resistant starch also modulate the intestinal microbiota of the host while providing nutritional value for growth performance. Microalgae have been considered as alternative feed ingredients to reduce the reliance on wild-caught fishmeal and fish oil in the diets of aquaculture species up to 50-75% replacement for corn/soymeal and fishmeal in the diet (Azaza et al., 2008; Benemann, 1992; Hussein, Dabrowski, El-Saidy, & Lee, 2013; Lazo, Dinis, Holt, Faulk, & Arnold, 2000; Olvera-Novoa, Domínguez-Cen, Olivera-Castillo, & Martínez-Palacios, 1998; Schrader, Green, & Perschbacher, 2011; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). They are ideal alternative feed ingredients because they can be easily cultivated and can be selected for high production of lipids and protein and may also modulate the intestinal microbiota of species of interest. *Arthrospira platensis* is a nutritionally enriched filamentous cyanobacterium with known anti-cancer and anti-oxidant properties in tilapia (*Oreochromis niloticus*) (Abdel-Tawwab & Ahmad, 2009; B. Belal, 2012; Hassan et al., 2017; Ibrahem & Ibrahim, 2014; Mahmoud, El-Lamie, Kilany, & Dessouki, 2018). *Schizochytrium limacinum* is a Docosahexaeonic acid (DHA) rich marine heterotroph that has successfully be used as a replacement for fish oil in red drum (*Sciaenops ocellatus*) (Perez-Velazquez, Gatlin, González-Félix, & García-Ortega, 2018), yellowtail (*Seriola rivoliana*) (Kissinger, García-Ortega, & Trushenski, 2016), and giant grouper (*Epinephelus lanceolotus*) (García-Ortega, Kissinger, & Trushenski, 2016). The combination of these two microbes was investigated as potential growth and health promoters in tilapia diets.

Tilapia (*Oreochromis sp.*) are the most widespread aquaculture species in the world due to their relative fecundity, omnivorous feeding habits, and tolerance of marginal growing conditions, making them ideal study species for alternative feed ingredients (FAO, 2016). Tilapia (*Oreochromis niloticus*) is the second most farmed fish group worldwide and over the past decade has quadrupled in production, largely due to their many characteristics conducive to aquaculture conditions as well as to the high marketability and relatively stable market prices (Ng and Romano, 2013). Production of tilapia in 2015 exceeded 5.7 million tons worldwide with a value of over $9 billion USD and has continued to subsequently increase (FAO, 2016). With an increasing demand for tilapia due to the increasing human population, producers must continue to provide high-quality tilapia filets with minimal input costs to meet this demand.

This study investigated the use of *Arthrospira fusiformis* and *Schizochytrium limacinum* as fishmeal and fish oil replacements to reduce feed cost while promoting the intestinal health of hybrid tilapia (*O. niloticus x O. mossambicus*) through the promotion of beneficial microbes.

Materials and methods

All animal procedures were conducted in accordance with the approval (protocol #17-007) from the Institutional Animal Care and Use Committee (IACUC) of the University of Hawaii, Honolulu, HI, USA.

Feeding trial

A feeding trial was conducted to study the replacement of fish meal, fish oil and soybean protein concentrate by algal meals of *Arthrospira platensis* and *Schizochytrium limacinum* in diets for tilapia. Five experimental diets were tested in fish with an initial weight of 0.9 ± 0.1 g. The replacement levels were: 0, 25, 50, 75 and 100% (Table 4.1). Each diet treatment was tested in triplicate in a water recirculation system with tanks of 90 L volume each at a density of 15 fish per tank. Fish were manually fed twice per day at 8 am and 2 pm for nine weeks. Fish feed intake and fish mortality were recorded daily, and a photoperiod of 12L:12D was applied. Water temperature, salinity and dissolved oxygen were measured in a daily basis in each tank with a portable multi-meter (Pro 2030, YSI Inc., USA) and water pH and total ammonia nitrogen measured weekly using a portable colorimeter (DR 900, Hach, USA). Water quality parameters averages were maintained at: temperature: 28.3 ± 0.2°C, salinity 0.1 ± 0.0 ppt, dissolved oxygen 6.5 ± 0.1 mg/L, pH 6.7 ± 0.3 and total ammonia nitrogen 0.0 ± 0.0 mg/L.

Intestinal microbiota sampling

Tilapia intestinal microbiota was sampled monthly and the intestinal samples from two fish per tank were pooled (three samples per treatment). The fishes were euthanized with 0.6 g l^{-1} Tricaine Methanesulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA), buffered with 0.12 g l^{-1} sodium bicarbonate in water originating from the corresponding rearing tank. Subsequently, fish were rinsed with 70% ethanol before dissecting out aseptically the gut under a dissection microscope. Whole gut samples were flash frozen in liquid nitrogen and stored individually at -80^{o}C until subsequent analyses. DNA extraction followed the protocol outlined by Yu and Morrison (2004) (Yu & Morrison, 2004). Microbial DNA was analyzed as outlined below.

16S rRNA gene sequencing analysis

Metagenomic analysis of the 16S rRNA V3 and V4 regions were conducted using the Illumina MiSeq system. The following primers were used prior to sequencing (in standard IUPAC nucleotide nomenclature): 16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC The quality of the reads was determined using FastQC and trimmed with Prinseq. QIIME was used for metagenomics quality control and taxon classification and quantification. DESeq2 was used for the differential abundance analysis of the operational taxonomic units (OTUs) identified. Krona charts (Ondov, Bergman, & Phillippy, 2011) were generated from the sequences using the MG-RAST server (Glass & Meyer, 2011) to illustrate the composition of intestinal microbiomes of tilapia fed different inclusion levels of moringa.

Histological Sampling of Intestines

Initial histological samples included 10 fish species after acclimation. At the end of the trial, two fish per tank were sampled for histological appraisal of the distal intestines. The following methods are from Rodiles et al. (2015). For light microscopy, the tissue samples were fixed in 10% formalin and transferred to 70% ethanol after 24h. Samples were dehydrated in graded ethanol concentrations prior to embedding in paraffin wax. In each specimen, multiple sections (5 µm) were stained with haematoxylin and eosin (H & E) and Alcian Blue-PAS to assess the mucosal fold length, intestinal perimeter ratio (arbitrary units; AU), intraepithelial leucocyte (IELs) levels and goblet cell abundance in the epithelium. IELs and goblet cells will be counted

across a standardized distance of 100 μm and then calculated by averaging the cell numbers from all samples within each treatment.

Statistical Analysis

The microbiota data were analyzed using DESeq2 analysis of deviance (ANODEV) and pair-wise comparisons between treatments. Significance was considered at $P < 0.05$. For diversity measures, Shannon Diversity Index was calculated for alpha diversity, and Bray-Curtis was used to compare beta diversity along with a principal coordinates analysis.

The histological parameters were analyzed by ANOVA using Mixed procedure of SAS (SAS 9.2, SAS Institute Inc., Cary, NC). Significant differences among treatments were assessed by Tukey's test. A significant level of P less than equal to 0.05 was used to declare difference.

Results

Intestinal Microbiota Metagenomic Results

Table 4.2 provides a summary of the species counts and Shannon Diversity Index. The Control group had the lowest index of diversity (2.248), while the 25% algae group had the highest (2.499) followed closely by the 100% algae group (2.453). The highest species count was in the 50% algae group with 916 species identified, while the 75% algae group had the lowest at 850. There were no significant differences between the beta diversity in each group, which is apparent in Figures 4.2 and 4.3.

In Figure 4.1, the top 30 bacterial families are summarized per sample and the full Krona Charts are shown in Figure 4.2. The four main Bacterial Phyla represented in all treatments included Actinobacteria, Proteobacteria, Firmicutes, and Fusobacteria. The most common bacterial families were the Mycobacterium, Streptomyces, and Singulisphaera for all samples but the 25% microalgae which had Cetobacterium as the second most dominant family and Nocardia as the third most dominant family behind Mycobacterium. Upon further investigation, the Mycobacterium Family was dominated by two species, *Mycobacterium pinnipedii*, a closely related species to the tuberculosis-causing *Mycobacterium tuberculosis*, and the non-tuberculosis causing, *Mycobacterium brasiliensis*.

The Principle Coordinate Analysis of the metagenomic results by Bacterial Families indicates clustering of the Control, 75%, and 100% Algae with the 25% and 50% as outliers (Figure 4.3).

Intestinal Histology Results

The intestinal histology results showed a significant difference in villi height between groups, including the initial sample ($p < 0.001$) and between treatments at the end of the trial ($p=0.0018$). The ALG25 treatment (150.77 µm ± 33.65) had significantly smaller villi than the initial samples (238.81µm ± 33.17). The ALG25 treatment (150.77 µm ± 33.65) also had significantly smaller villi than the ALG0 samples (247.29µm ± 89.76). There was no significant difference ($p > 0.05$) between goblet cell counts and intraepithelial leucocyte (IEL) levels between treatments. There were increased IEL levels in the ALG50 and ALG75 treatments; however, it was not significantly different from the control. Additionally, there were increased goblet cells in the ALG50 treatment, suggesting minor inflammation in the tissues.

Discussion

To date, few intestinal microbiota studies have been conducted on organisms fed microalgae, including; gilthead seabream (*Sparus aurata*) fed two types of algae (*Tetraselmis chuii* and *Phaeodactylum tricornutum*) and the probiotic *Bacillus subtilis* (Esteban et al., 2012); rainbow trout (*Oncorhynchus mykiss*) fed *Schizochytrium limacinum* (Lyons, Turnbull, Dawson, & Crumlish, 2017), and another study involved feeding mice one of three different algal treatments (*Nostoc commune, Spirulina platensis,* or *Afanizominon flos-aquae*) (Rasmussen, Martínez, Lee, & Walter, 2009). The study by Cerezuela, et al. (2012) found significant reduction in the microbial diversity and total numbers with increasing levels of algal inclusion in the diet; whereas, Lyons, et al. (2017) and Rasmussen, et al. (2009) did not find significant differences in total microbial counts, but Rasmussen, et al. (2009) did find significant differences between the microbial community in the control vs. experimental group fed *N. commune*.

Based on the results of this study, microalgae can be incorporated to replace up to 100% of the fishmeal and fish oil in tilapia diets without negative effects on growth or health of the fish. It has been well documented that feed alters the intestinal microbiome within fishes, particularly by altering the carbohydrate content of the diet (Haygood & Jha, 2018; Merrifield & Ringø, 2014; Sukanta K Nayak, 2010; Ringø et al., 2010). According to the Shannon Diversity Index results, with increasing microalgae inclusion in the diet there was an increase in diversity with the exception of the 75% microalgae group; therefore, microalgae inclusion in the diet as a fishmeal and fish oil replacement alters the intestinal microbiota of tilapia.

The four main bacterial Phyla represented in all treatments included Actinobacteria, Proteobacteria, Firmicutes, and Fusobacteria, which supports previous studies regarding the dominant phyla in fish microbiomes based on metagenomic analyses (Tarnecki, Burgos, Ray, & Arias, 2017). The most common bacterial families were the Mycobacterium, Streptomyces, and Singulisphaera for all samples but the 25% microalgae which had Cetobacterium as the second most dominant family and Nocardia as the third most dominant family behind Mycobacterium. Cetobacterium have been reported in tilapia intestinal microbiota previously (Larsen, Mohammed, & Arias, 2014; Pedrotti et al., 2015); however, their role in the intestine is unclear.

The Mycobacterium Family was dominated by two species, *Mycobacterium pinnipedii*, a closely related species to the tuberculosis-causing *Mycobacterium tuberculosis*, and the non-tuberculosis causing, *Mycobacterium brasiliensis*. To the author's knowledge, this is the first case of these species reported in tilapia. While *Mycobacterium pinnipedii* is a pathogen of seals, these species were found in abundance across all diet types and did not seem to have deleterious effects on growth parameters, survival, and health parameters measured in this study.

The family Bacillaceae was significantly different between treatments ($p < 0.05$) with the highest levels in the ALG 100 (2062 OTUs) and ALG 50 (1757 OTUs). Bacillaceae includes several probiotic species tested on tilapia, including: *Bacillus subtilis* (Adeoye et al., 2016; Efendi & Yusra, 2014; Essa, El-Serafy, & El-Ezabi, 2010; He, Zhang, Xu, Yalin, et al., 2013; He, Zhang, Xu, Yang, et al., 2013; Iwashita, Nakandakare, Terhune, Wood, & Ranzani-Paiva, 2015; Kathia, Cienfuegos Martinez, del Carmen, Monroy Dosta Maria, Aida, Hamdan Partida, Jorge, Castro Mejia, Feliz, Aguirre Garrido Jose, Amadeo, 2018; Ng, Kim, Romano, Koh, & Yang, 2014; Rahman et al., 2009; Rodiles et al., 2015; Rurangwa et al., 2009; Soltan & El-Laithy, 2008; Standen et al., 2015; Telli et al., 2014; Zhou, Tian, Wang, & Li, 2010); *Bacillus pumilus* (Adeoye et al., 2016; Aly, Mohamed, & John, 2008); and *Bacillus licheniformis* (Adeoye et al., 2016; Ng et al., 2014). These three *Bacillus* species are confirmed probiotics of tilapia species and their benefits include improving both growth and health parameters. With increasing levels of *Arthrospira fusiformis* and *Schizochytrium limacinum* in the diets of tilapia, the Bacillaceae family has increased which may promote the health and growth of tilapia without having to include these microbes as separate probiotic ingredients.

While the intestinal morphology of the tilapia was significantly different between the ALG25 treatment and the control, there is no significant difference in the increased algal inclusion levels up to 100% replacement of fishmeal and fish oil. There was no significant difference in the goblet cell and intraepithelial leucocyte levels between all treatments.

In conclusion, *Arthrospira fusiformis* and *Schizochytrium limacinum* inclusion into the diets of tilapia significantly increased the Bacteriaceae family in the tilapia intestinal microbiota, suggesting that microalgae inclusion in the diet may beneficially affect tilapia. More research into other histological parameters will help to determine if *Arthrospira fusiformis* and *Schizochytrium limacinum* have beneficial effects on the health of tilapia.

Table 4.1 Ingredient composition and nutrient content of diets fed in the microalgae study

	Diets (g/kg)				
Ingredient	**ALG-0**	**ALG-25**	**ALG-50**	**ALG-75**	**ALG-100**
Fish meal	400	300	200	100	0
Fish oil	76.06	57.05	38.03	19.02	0
Arthorspira	0	165.47	330.99	496.42	661.9
Schizochytrium	0	40.48	80.98	121.47	161.97
Soy Protein Concentrate	195.58	146.69	97.75	48.9	0
Dextrin	262.37	223.31	184.24	145.18	106.12
Alginate	20	15	10	5	0
Di-calcium phosphate	10	10	10	10	10
Vitamin premix	20	20	20	20	20
Mineral premix	10	10	10	10	10
Lysine	0	3	6	9	12
Methionine	0	2	4	6	8
Taurine	6	7	8	9	10
Total	1000.0	1000.0	1000.0	1000.0	1000.0

Table 4.2 Number of reads, species counts, and Shannon Diversity Index values per sample.

Sample	Total Reads	Species Count	Shannon Diversity Index
Control	102,963	871	2.248
25% Algae	118,261	901	2.499
50% Algae	116,228	916	2.405
75% Algae	106,699	850	2.274
100% Algae	96,283	874	2.453

Figure 4.1 Top 30 Genus classification results per sample. Samples are labeled as follows: ALG0 = control, ALG25 = 25% algae, ALG50 = 50% algae, ALG75 = 75% algae, ALG100 = 100% algae.

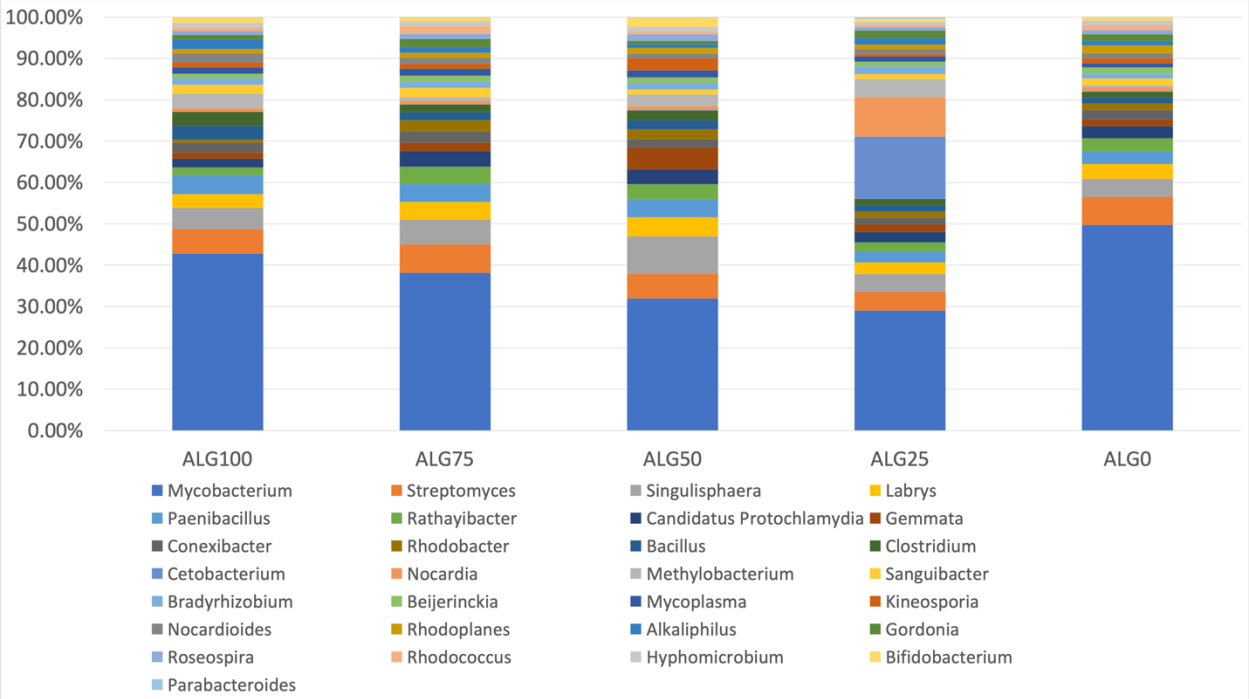
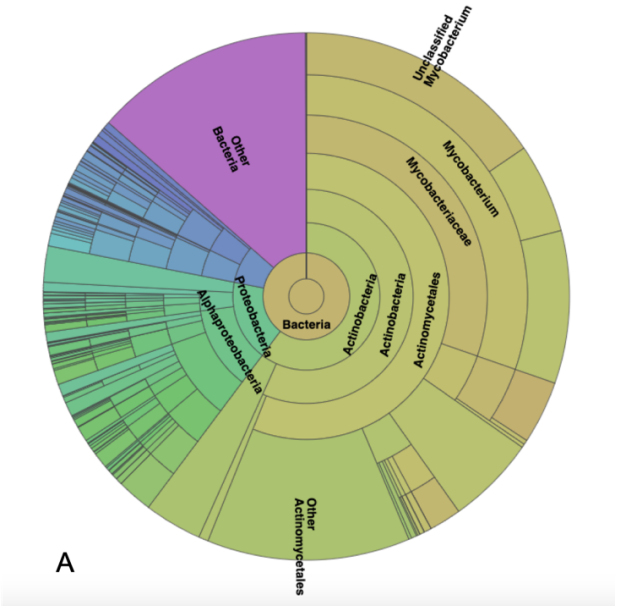
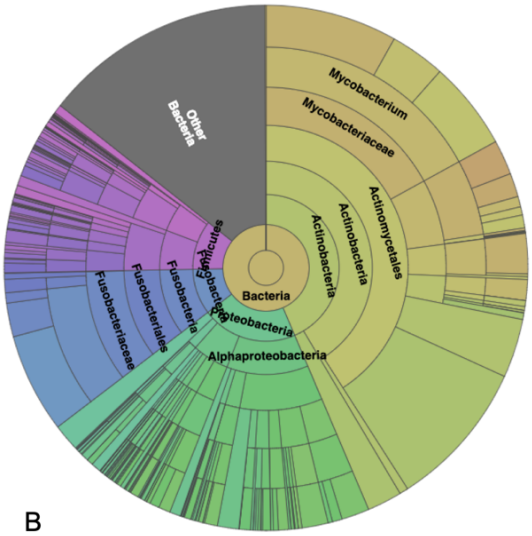
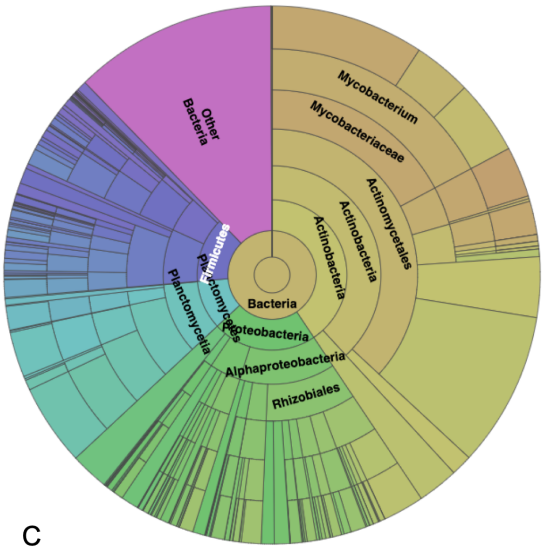
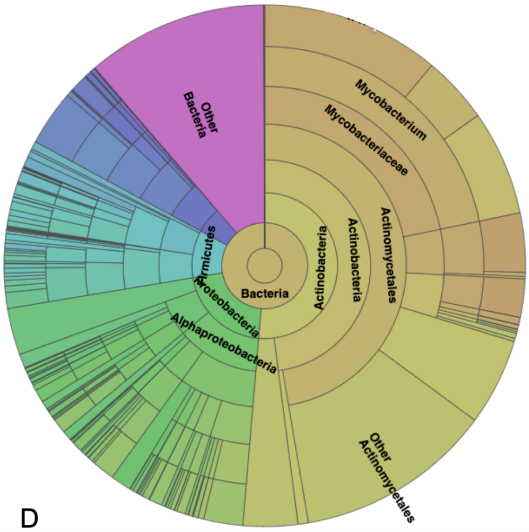


Figure 4.2 Krona charts of bacterial classifications by sample.









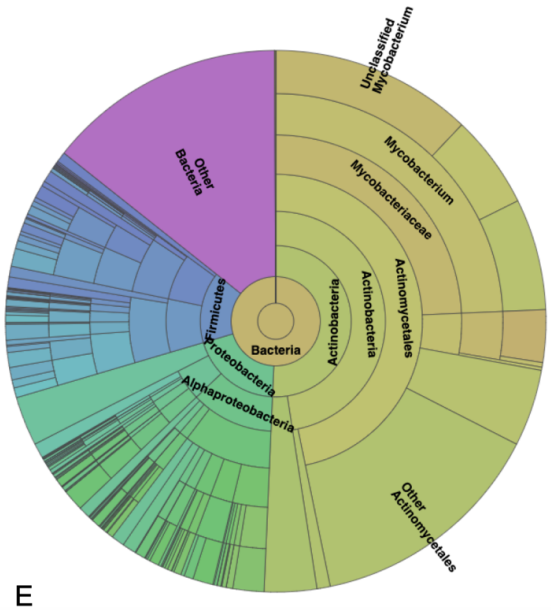


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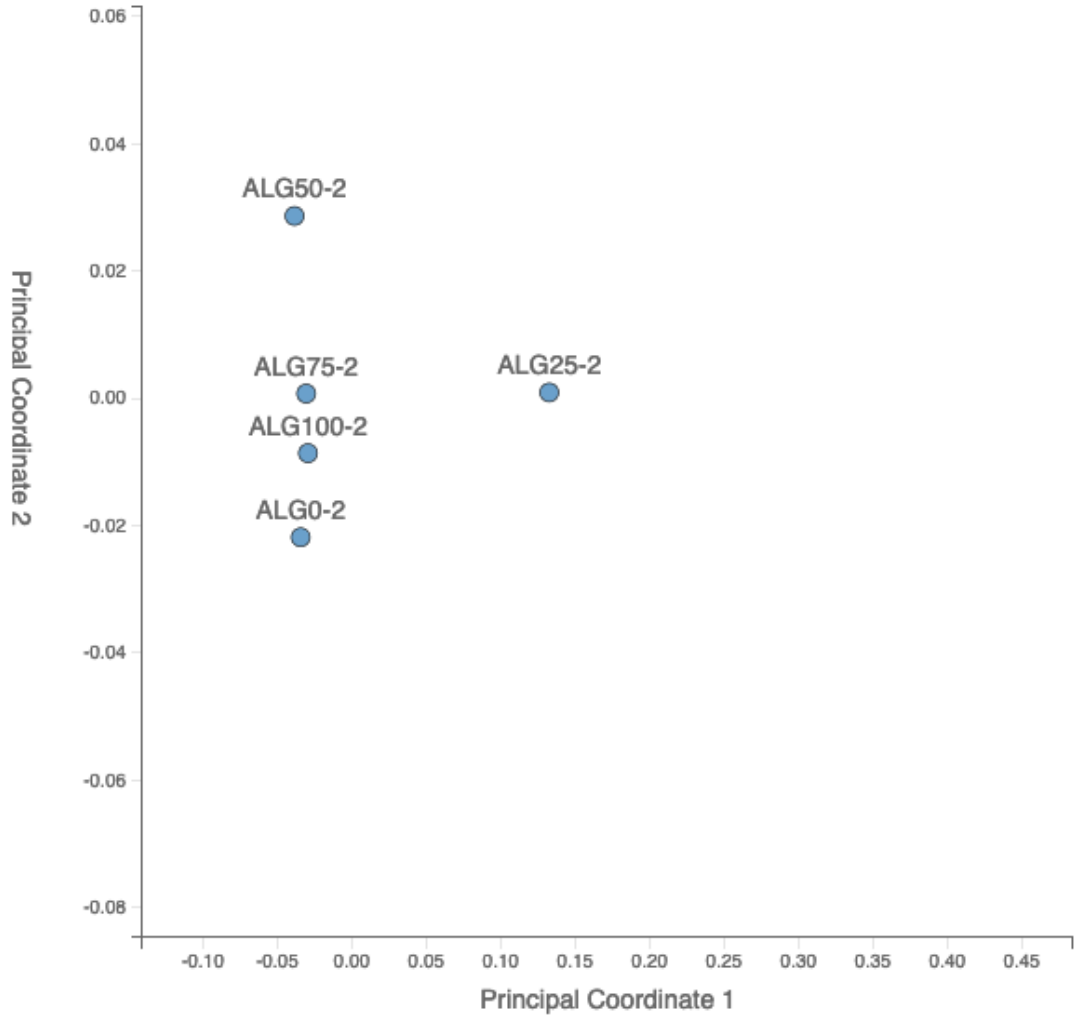
Figure 4.3 Principle Coordinate Analysis (PCoA) of the normalized relative abundance of all samples based on family-level classifications. Samples are labeled as follows: ALG0-2 = control, ALG25-2 = 25% algae, ALG50-2 = 50% algae, ALG75-2 = 75% algae, ALG100-2 = 100% algae.



CHAPTER 5 DISCUSSION

The intestinal microbiome is a dynamic ecosystem that is highly regulated by the host (de Blas et al., 2010). Changes in endocrine signaling pathways are driven by the enteric nervous system and allow fish to adapt their gastrointestinal (GI) characteristics, including the composition of the microbiome (de Blas et al., 2010; D. Merrifield & Ringø, 2014). While fermentation is important in other vertebrates, particularly humans and ruminants, it is not well understood in fish. Many species of bacteria are present in the gastrointestinal tract and are known to break down non-digestible carbohydrates producing metabolites that are important to the host, such as short-chain fatty acids. Therefore, it is assumed that these bacteria play a similar role in fish digestion as they do in mammalian digestion (Gibson & Roberfroid, 1995; D. Merrifield & Ringø, 2014).

The composition of the gastrointestinal microbiota is highly dependent on host species-specific parameters including; anatomy, endogenous inputs of digestive secretions, pH, osmolality, redox potential, compartment size and structure, and passage rates and residence times (D. Merrifield & Ringø, 2014). The anatomy of the GI tract varies widely between species and is primarily dependent on the environment and feeding habits. Tilapia (*Oreochromis spp.*) are primarily freshwater omnivores with a digestive tract composed of a mouth, esophagus, stomach, midgut, and hindgut. The intestinal tract (midgut and hindgut) are divided into the following sections hepatic loop, proximal major coil, gastric loop, distal major coil, and terminal segment (Smith, Smith, Tengjaroenkul, & Lawrence, 2000). Each segment contains autochthonous (resident) and allochthonous (transient) microbial communities that affect the health and survival of the host (D. Merrifield & Ringø, 2014).

The GI microbiome of fish is initially colonized at hatching and can change throughout the life cycle of the fish, particularly as their feeding preferences change (Giatsis et al., 2014; Pérez et al., 2010). Herbivorous fish contain the most diverse microbiome, as seen in other vertebrate species. For tilapia, their intestinal microbiome is highly diverse due to their omnivorous nature with a preference for feeding on plant and algal material. The Proteobacteria and Firmicutes Phyla tend to dominate the fish GI microbiome (Haygood & Jha, 2018; Mansfield et al., 2010; Zheng et al., 2018). The “normal” microbiome of fish species are autochthonous species that cause no damage to, and possibly even benefit, their host. Known benefits of microbiota include their ability to regulate digestion and energy homeostasis, prevent colonization of infectious

agents, help maintain the mucosal immunity of their host, promote angiogenesis, and are known to regulate 212 genes in zebrafish (Cahill, 1990; Merrifield & Ringø, 2014; Nayak, 2010; Nicholson et al., 2012).

In addition to the host-specific parameters listed previously, the intestinal microbiome of fish is dependent on environmental factors and the diet of the fish. Environmental factors are known to affect the growth and development of the intestinal microbiome, including seasonal changes in temperature, daily fluctuations in salinity, and alterations to the environment. These factors may highly influence the colonization of the intestinal microbiome and help determine the mature microbiome of the adult fish (Giatsis et al., 2014; D. Merrifield & Ringø, 2014; Pérez et al., 2010).

The two most highly investigated areas for modulation of the intestinal microbiota are probiotics and prebiotics in diet inclusion (Haygood & Jha, 2018; D. Merrifield & Ringø, 2014). Probiotics include live microorganisms that have different beneficial characteristics to their host (Nayak, 2010; Tuan, Duc, & Hatai, 2013). The vast majority of probiotics studied to date are Lactic Acid Bacteria, but the other main probiotics in aquatic animals include: Aeromonas, Alteromonas, Enterobacter, Flavobacterium, Pseudomonas, Pseudoalteromonas, Phaecobacter, Roseobacter, Shewanella, Vibrio, Arthrobacter, Bacillus, Clostridium, Micrococcus, Debaryomyces, and Saccharomyces species (D. Merrifield & Ringø, 2014). Probiotics have been shown to enhance survival, development, nutrition, and disease resistance in the host species.

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already residing in the colon, and thus attempt to improve host health (Gibson & Roberfroid, 1995). Due to the possible environmental issues with probiotics, many companies have investigated the use of prebiotics to promote the growth of beneficial autochthonous bacterial species present in the fish. The most common prebiotics used are inulin, various oligosaccharides, GroBiotic®, and Previda™. Prebiotics are required by bacteria that produce short-chain organic acids that have beneficial effects on the host. By incorporating prebiotics into the feed, the GI microbiota can be modulated to promote beneficial species, in turn providing the following benefits to the host: modulation of blood lipid levels; GI/systemic immunomodulation; energy for intestinal proliferation; improved intestinal barrier function; reduced pH which aids in mineral absorption

and general nutritional support; and enhancing pathogen resistance, reducing toxic microbial metabolites, and suppressing intestinal inflammation (D. Merrifield & Ringø, 2014).

The GI microbiome of tilapia is highly varied depending on species and location. Currently, very little is known of the "normal" tilapia microbiota present in the gastrointestinal tract. Early investigations of the intestinal microbiota of tilapia species utilized culture-dependent techniques and were limited to identification of the most common and easily cultured species present. As reviewed by Cahill (1990), the bacteria present in the intestinal tract of tilapia included *Pseudomonas sp.*, *Virbio sp.*, *Aeromonas sp.*, *Enterobacteriaceae sp.*, and other unidentified species. A study by Molinari et al. (2003) examined the microflora in mature tilapia cultured in a semi-intensive system. They found the following bacteria present: *Aeromonas hydrophila, A. veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, Flavimonas oryzihabitans and Plesiomonas shigelloides*. Another study by Pakingking, Palma, & Usero (2015) cultured microbial species present in mature tilapia grown in earthen ponds. They identified the following heterotrophic aerobic bacteria in the tilapia intestinal tract: *Aeromonas hydrophila, A. sobria, Bacillus sp., Citrobacter koseri, Edwardsiella tarda, Edwardisella hoshinae, Enterobacter cloacae, Klebsiella oxytoca, Pasteurella pneumotropica, Photobacterium damselae, Plesiomonas shigelloides, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas luteola, Pseudomonas putida, Shewanella putrefaciens, Staphyloccocus sp., Vibrio cholera, V. fluvialis, V. vulnificus,* and unidentified gram-negative rod species.

Recent advances in the identification of microbiota using molecular techniques have expanded our knowledge of fish microbiota ten-fold, with particular attention given to salmonids (Merrifield & Ringø, 2014; Nayak, 2010). Though many molecular studies summarized below investigate the modulation of intestinal microbiota of tilapia, to the authors' knowledge, the first attempt to characterize the core microbiota of wild cichlid's GI tract was only recently completed by Baldo, Riera, Tooming-Klunderud, Albà, & Salzburger (2015). Using 16S rRNA pyrosequencing of microbial DNA samples from ten cichlid species, they determined the core bacterial taxa present in at least 80% of the individuals. The species identified represent a diverse group of phyla including *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Planctomcetes*, and *Verrucomicrobia*. The representative taxon from these phyla included: *Cetobacterium somerae, Clostridium perfringens, Plesiomonas shigelloides, Turicibacter sp., Clostridium XI sp., Aeromonas sp., Neisseriacea, Lachnospiraceae, Clostridiales, Clostridiaceae, Gemmataceae,*

Acromobacter sp., Bacillus sp., and *Pirellulaceae* (Baldo et al., 2015). This study can be used as a baseline for intestinal modulation of microbiota in aquaculture.

Yeast such as *Saccharomyces cerevisiae* are important heterotrophic fermenters in the GI tract and have been proposed as a probiotic for tilapia (Abdel-Tawwab et al., 2008; Ayyat et al., 2014; He et al., 2009; Lara-Flores et al., 2003; D. Merrifield & Ringø, 2014). Several yeast species, including *Kloeckera apiculata, Cadida sp., Metcschnikowia sp.,* and *Rhodotorula sp.*, have been described in finfish other than tilapia (Gatesoupe, 2007; D. Merrifield & Ringø, 2014). Yeasts are known immuno-stimulants in fish and may promote growth and development in older fish (Gatesoupe, 2007). However, despite understanding the importance of yeast in the GI tract of fishes, no studies have investigated the naturally occurring presence or function of eukaryotic autochthonous intestinal microbiota in tilapia.

The purpose of this research was to determine the effects of alternative feed ingredients on the GI microbiota of hybrid tilapia (*Oreochromis niloticus* x. *O. mossambicus*). The alternative feed ingredients investigated in these studies were moringa (*Moringa stenopetala*), cassava (*Manicot esculenta*), and microalgae (*Arthrospira fusiformis* and *Schizochytrium limacinum*) to replace fishmeal, corn, and fishmeal/fish oil respectively. Moringa can be incorporated up to 12%, cassava up to 26.25%, and microalgae up to 83.4% of the diet of hybrid tilapia without negatively affecting the growth and production of the fish. By incorporating these alternative feed ingredients on a large scale, producers can reduce overall costs without incurring negative effects on production of hybrid tilapia.

The overall intestinal microbiota were significantly altered with moringa and cassava inclusion in the diets. Previous research stated that Proteobacteria, Firmicutes, Planctomycetes, and Actinobacteria were the vast majority of bacteria present in the tilapia samples (Rodiles et al., 2015; Tarnecki, Burgos, Ray, & Arias, 2017b) and this work was supported in our research. The microalgae also had a large representation from Fusobacteria in the metagenomic results. While the same phyla were represented across trials, the bacterial families were varied. The top ten families for each trial are presented in Table 5.1. The families represented predominantly across all treatments were: Microbacteriaceae, Rhodobacteraceae, Mycobacteriaceae, Isosphaeraceae, Xanthobacteraceae, and Microbacteriaceae.

With moringa inclusion, the Oxalobacteraceae and Comamonadaceae families were significantly higher and the Proteobacteraceae family was significantly reduced, suggesting there may be anti-inflammatory effects of *Moringa stenopetala* similar to the known effects of *Moringa oleifera*. With cassava inclusion, Microbacteriaceae, Rhizobiaceae, and Methylobacteriaceae were significantly higher, and Streptococcaceae and Xanthobacteracea were significantly reduced. The increase of the Methylobacteriaceae family in the tilapia intestinal microbiota suggest that cassava inclusion in the diet may beneficially affect tilapia. Additionally, the reduction in levels in Streptococcaceae in the intestine of tilapia suggests that *M. esculenta* may reduce pathogenic load in farmed tilapia. Finally, microalgae inclusion increased the levels of Bacillaceae, a known probiotic family which suggests that microalgae inclusion in the diet may beneficially affect tilapia. The most concerning find in this study was the presence of *Mycobacterium pinnipedii*, a closely related species to the tuberculosis-causing *Mycobacterium tuberculosis* across all microalgae treatments and the control. To the author's knowledge, this is the first case of these species reported in tilapia. While *Mycobacterium pinnipedii* is a pathogen of seals, these species were found in abundance across all diet types and did not seem to have deleterious effects on growth parameters, survival, and health parameters measured in this study.

The results of these studies provide some of the first research into the effects of these alterative feed ingredients on the intestinal microbiota of hybrid tilapia. While it is well documented that food ingredients alter the intestinal microbiota of humans (Foxx-Orenstein & Chey, 2012; Gerritsen, Smidt, Rijkers, & De Vos, 2011; Gibson & Roberfroid, 1995; Hooper et al., 2002), more work is required to study the effects of feed ingredients on the intestinal microbiota and overall health of tilapia.

Future Research

Based on the results of these studies, the following are suggestions for future research: investigation of other alternative feed ingredients to promote growth, health, and modulation of the intestinal microbiome of tilapia; investigations into the mode of action for colonization and maturation of the intestinal microbiome; and investigations into the mode of action for the intestinal microbiome effects on health and growth of fish, particularly tilapia. According to Lescak & Milligan-Myhre (2017), teleost species, like tilapia, are ideal model organisms to study the evolution and interactions between the host and their microbiome. They state "the extensive variation in the physiology, ecology, and natural history of fish enriches studies of the evolution

and ecology of host-microbe interactions. They share physiological and immunological features common among vertebrates, including humans, and harbor complex gut microbiota, which allows identification of the mechanisms driving microbial community assembly. Their accelerated life cycles and large clutch sizes and the ease of sampling both internal and external microbial communities make them particularly well suited for robust statistical studies of microbial diversity. Gnotobiotic techniques, genetic manipulation of the microbiota and host, and transparent juveniles enable novel insights into mechanisms underlying development of the digestive tract and disease states. Many diseases involve a complex combination of genes which are difficult to manipulate in homogeneous model organisms. By taking advantage of the natural genetic variation found in wild fish populations, as well as of the availability of powerful genetic tools, future studies should be able to identify conserved genes and pathways that contribute to human genetic diseases characterized by dysbiosis."

While probiotics have been extensively studied in tilapia, two of the main challenges of the use of probiotics in aquaculture are the costs and shelf-life of the probiotic treatments. Since the bacterial species of interest must be alive at the time of consumption, they are difficult to transport across long distances and may lose effectiveness if not stored at ideal temperatures (D. Merrifield & Ringø, 2014). Prebiotics are an effective alternative at promoting beneficial microbes in the host; however, they are an additional cost to the farmer. By using alternative feed ingredients as prebiotics, the costs of the aquaculture feed can be reduced while the effects of the promotion of beneficial bacterial species can be maintained. Therefore, research into the use of commercially available alternative feed ingredients should be continued. The research should focus particularly on the growth, health, and economic effects of the proposed feed ingredients.

While many species have been labeled as "probiotic" and "beneficial microbes," there is still a lack of knowledge into the mode of action of these species on the host. The colonization and maturation of the intestinal microbial ecosystem is dependent on the rearing conditions (Dehler, Secombes, & Martin, 2017; Waché et al., 2006), genetics of the host (Lescak & Milligan-Myhre, 2017), and feed ingredients, including probiotics and prebiotics (Dimitroglou et al., 2011; Haygood & Jha, 2018; D. Merrifield & Ringø, 2014; Ringø et al., 2010). Because this dynamic environment is difficult to replicate in laboratory conditions, very little is known of the colonization and maturation of intestinal microbes.

Finally, as stated previously, the known benefits of microbiota include their ability to regulate digestion and energy homeostasis, prevent colonization of infectious agents, help maintain the mucosal immunity of their host, promote angiogenesis, and are known to regulate 212 genes in zebrafish (Cahill, 1990; Merrifield & Ringø, 2014; Nayak, 2010; Nicholson et al., 2012). While the effects of probiotics are recorded in the host, little is known of the exact modes of action of these microbes. Gene expression studies can be used to determine what host genes are affected by the application of species of interest and classify bacterial species into different groups based on their beneficial effects. Additionally, if the host intestinal environment could be replicated in vitro, more work can be done on determine the modes of action and identification of new beneficial species.

The overall goal is to reduce the production costs of farmers to promote the production of high-quality fish protein for an ever-increasing human population. Once the effects of intestinal microbes are better understood, they can be modulated to increase the growth and health benefits to the aquaculture species of interest to meet this goal.

Table 5.1 Summary of the top ten bacterial families found in each study; moringa, cassava, and microalgae. Each family and average OTUs are listed.

Moringa		**Cassava**		**Microalgae**	
Family	**OTUs**	**Family**	**OTUs**	**Family**	**OTUs**
Microbacteriaceae	5749	Gemmataceae	20832.2	Mycobacteriaceae	25379
Sphingomonadaceae	1809	Streptococcaceae	14352.4	Streptomycetaceae	4252
Chitinophagaceae	1797	Rhizobiaceae	4720.6	Isosphaeraceae	4197
Cytophagaceae	1658	Rhodobacteraceae	3935.8	Xanthobacteraceae	4085
Rhodospirillaceae	1504	Mycobacteriaceae	3901.4	Microbacteriaceae	3541
Caulobacteraceae	1348	Fusobacteriaceae	3892.4	Paenibacillaceae	2974
Saprospiraceae	1159	Isosphaeraceae	3633.6	Rhodobacteraceae	2401
Sphingobacteriales	1126	Methylobacteriaceae	3542.6	Clostridiaceae	2223
Bacteroidetes Family	1096	Xanthobacteraceae	3253	Pseudonocardiaceae	2080
Rhodobacteraceae	864	Micrococcaceae	1743.4	Nocardiodiaceae	1852

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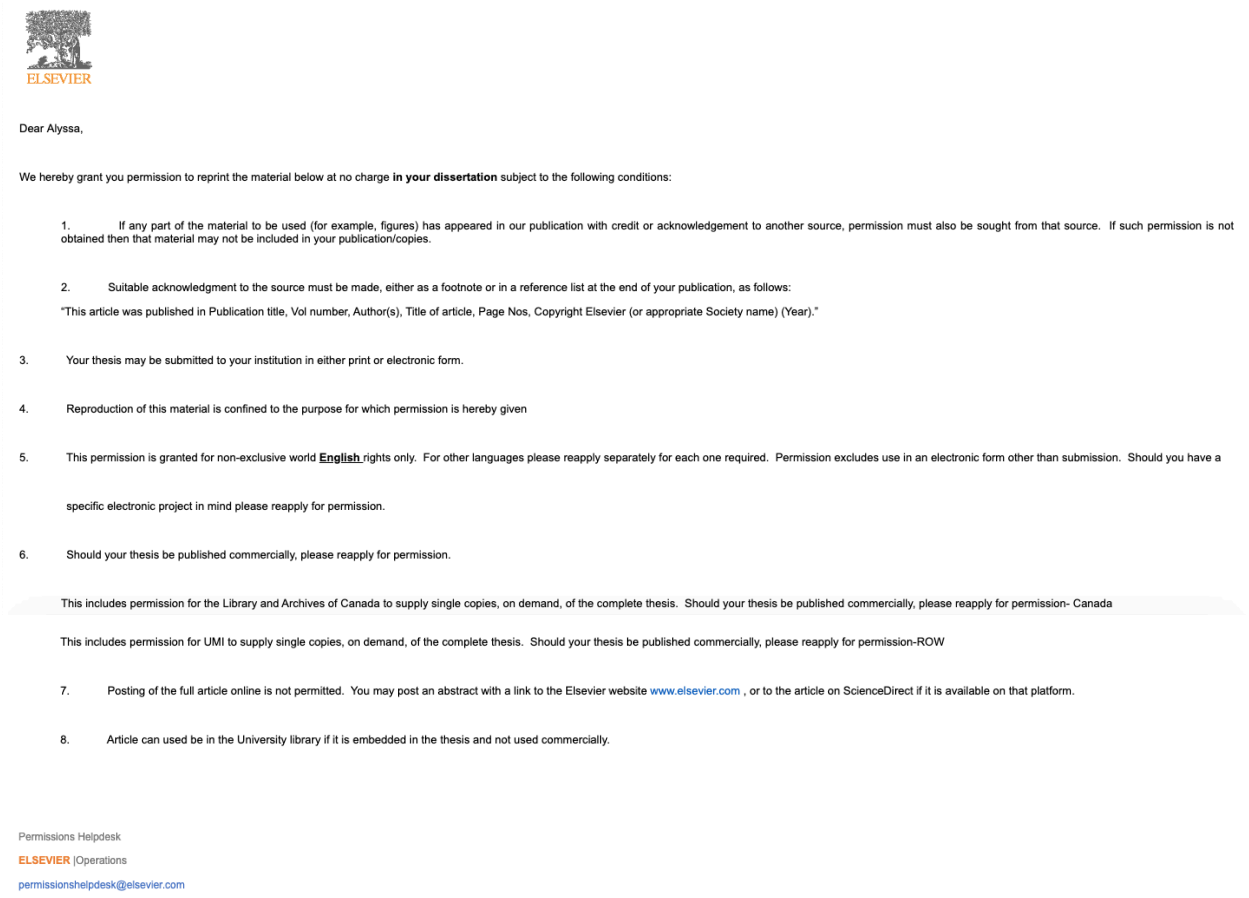
Appendix 1: Letter of Consent to Publish from Reviews in Aquaculture



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